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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371		Attorney Docket Number 5525-0044.10
International Application No., PCT/US00/04349		International Filing Date 18 February 2000
Title of Invention POLYMORPHIC DNA FRAGMENTS AND USES THEREOF		U.S. Application No. (if known, see 37 CFR §1.5) 09/914101
Applicant(s) for DO/EO/US Sydney Brenner		Priority Date Claimed 22 February 1999
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. §371.		
2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. §371.		
3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).		
4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.		
5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2))		
a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.		
c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).		
6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)).		
7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))		
a. <input checked="" type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).		
b. <input type="checkbox"/> have been transmitted by the International Bureau.		
c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.		
d. <input type="checkbox"/> have not been made and will not be made.		
8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).		
9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).		
10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).		
Items 11. to 16. below concern document(s) or information included:		
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR §1.97 and §1.98.		
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR §3.28 and §3.31 is included.		
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.		
<input type="checkbox"/> A SECOND or SUBSTITUTE preliminary amendment.		
14. <input type="checkbox"/> A substitute specification.		
15. <input type="checkbox"/> A change of Power of Attorney and/or Address letter.		
16. <input type="checkbox"/> Other items or information:		

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U.S. Application No. (if known, see 37 CFR §1.5) <div style="font-size: 24pt; font-weight: bold; text-align: center;">09/914101</div>	International Application No. <div style="font-weight: bold; text-align: center;">PCT/US00/04349</div>	Attorney's Docket No. <div style="font-weight: bold; text-align: center;">5525-0044.10</div>
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17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR §1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **\$1,000.00**

International preliminary examination fee (37 CFR §1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **\$ 860.00**

International preliminary examination fee (37 CFR §1.482) not paid to USPTO but international search fee (37 CFR §1.445(a)(2)) paid to USPTO **\$ 710.00**

International preliminary examination fee paid to USPTO (37 CFR §1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$ 690.00**

International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **\$100.00**

CALCULATIONS PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =	\$860.00	
Surcharge of \$130 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claim priority date (37 CFR §1.492(e))	\$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	20 - 20	0	x \$ 18.00	\$ 00.00	
Independent Claims	7 - 3	4	4 x \$ 80.00	\$320.00	
Multiple Dependent Claim(s) (if applicable)			+ \$270.00	\$ 00.00	
TOTAL OF ABOVE CALCULATIONS =				\$1310.00	

☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.

SUBTOTAL =	\$1310.00	
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Processing fee of \$130 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR §1.942(f))

Fee for recording encl. assignment (37 CFR §1.21(h)). The assignment must be accompanied by recordation cover sheet (37 CFR §3.28) \$40 per prop	\$	
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TOTAL FEES ENCLOSED =	\$1310.00	
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Amount To Be refunded	\$	
Amount to Be charged	\$	

a. ☐ A check in the amount of \$* to cover the filing fees is enclosed.

b. ☒ Please charge Deposit Account No. 50-0665 in the amount of \$1310.00 to cover the above fees. This sheet is provided in triplicate.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0665.

NOTE: Where an appropriate time limit under 37 CFR §1.494 or §1.495 has not been met, a petition to revive (37 CFR §1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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09/914101 040402
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I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as Express Mail Post Office to Addressee, Express Mail Label. No. EL 889535258 US in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C., 20231, on:

Date: August 21, 2001 By: Lynnea B. Anderson

DOCKET No.: 5525-0044.10

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF: Sydney Brenner

SERIAL NO.: not yet assigned

FILED: 20 August 2001

FOR: **POLYMORPHIC DNA FRAGMENTS AND
USES THEREOF**

EXAMINER: not yet assigned

ART UNIT: not yet assigned

Preliminary Amendment

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above application, please amend the application as follows:

In the Specification:

At page 1, line 1 of the specification, preceding "FIELD OF THE INVENTION", please insert the following paragraph:

This application is a U.S. National Stage filing under 35 U.S.C. §371 of PCT Appn. No. PCT/US00/04349, which claims priority to U.S. Provisional Appn. No. 60/121,023, filed February 22, 1999, and to U.S. Provisional Appn. No. 60/158,483, filed October 8, 1999, all of which are hereby incorporated by reference in their entirety.

In the Claims:

Please replace claims 1-12, 15-16 and 18 with the rewritten claims below, and add claims 19-20, as follows. Also enclosed, starting on a separate page following this response, is a marked copy of the presently amended claims showing all changes relative to the previous version.

1. A nucleic acid reference library derived from pooled DNA from at least two sources, said library comprising a heterogeneous mixture of nucleic acid fragments, wherein
 each said fragment (a) is a portion of a polymorphic subregion of a polymorphic consensus sequence derived from said pooled DNA or (b) is derived from a non-polymorphic subregion,
 each of said polymorphic subregions is bounded by first restriction sites and
 comprises an internal polymorphic restriction site which is different from said first site;
 said polymorphic consensus sequence is the theoretical sequence obtained by (i) aligning said pooled DNA to provide maximum homology, and (ii) projecting each of said restriction sites onto said sequence; and
 said library is enriched for fragments of type (a) relative to type (b).

2. A nucleic acid reference library according to claim 1, wherein at least a subpopulation of said nucleic acid fragments further comprise oligonucleotide tags, and different nucleic acid fragments are linked to different oligonucleotide tags.

3. The nucleic acid reference library according to claim 2, wherein said fragments are contained within a replicable vector.

4. The nucleic acid reference library according to claim 2, wherein said oligonucleotide tags comprise oligonucleotides of the form:

$$S_1 S_2 S_3 \dots S_n$$

wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

5. A composition comprising subpopulations of microparticles, wherein each subpopulation comprises at least one microparticle, said microparticle comprising a polymorphic probe, wherein the polymorphic probe of each subpopulation is different from those of the other subpopulations, and comprises a portion of a polymorphic subregion of a polymorphic consensus

sequence as recited in claim 1.

6. The composition of claim 5, wherein each of said subpopulations further comprises a oligonucleotide tag, and different subpopulations comprise different tags.

7. The composition according to claim 6, wherein each said oligonucleotide tag is positioned between said microparticle and said polymorphic probe.

8. The composition according to claim 6, wherein each said oligonucleotide tags comprises an oligonucleotide of the form:

$$S_1S_2S_3 \dots S_n$$

wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

9. An array comprising a solid support having defined regions on the surface thereof, wherein each region comprises a different polymorphic probe, and wherein each of said polymorphic probes comprises a portion of a polymorphic subregion of a polymorphic consensus sequence as recited in claim 1.

10. The array of claim 9, wherein each of said regions further comprises an oligonucleotide tag, and different subpopulations comprise different tags.

11. The array of claim 10, wherein each said oligonucleotide tag is positioned between said surface and said polymorphic probe.

12. The array according to claim 11, wherein each said oligonucleotide tag comprises an oligonucleotide of the form:

$$S_1S_2S_3 \dots S_n$$

wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from

3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

13. A nucleic acid reference library of genomic DNA from a plurality of individuals comprising:
a heterogeneous mixture of restriction fragments of a first restriction endonuclease;

wherein said restriction fragments from the same locus contain members having one or more restriction site polymorphisms with respect to a second restriction endonuclease, the number of such members forming a proper subset of the total number of restriction fragments from said locus.

14. A nucleic acid reference library of genomic DNA from a plurality of individuals comprising:
a heterogeneous mixture of restriction fragments of a first restriction endonuclease;

wherein said restriction fragments from the same locus contain at least one member having one or more restriction site polymorphisms with respect to a second restriction endonuclease and at least one member without said restriction site polymorphism.

15. A method of making a reference library comprising a mixture of heterogeneous nucleic acid fragments, comprising:

digesting pooled nucleic acid comprising first restriction sites with a first restriction endonuclease to produce a mixture of restriction fragments;

forming a first population of single stranded nucleic DNA fragments from a first subpopulation of said restriction fragments, wherein said first subpopulation of restriction fragments comprises a second restriction site which is different from said first restriction site;

forming a second population of single stranded DNA fragments from a second subpopulation of said restriction fragments, wherein said second subpopulation of said restriction fragments do not contain said second restriction site,

and wherein said first single stranded DNA fragments are complementary to said second single stranded DNA fragments when said single stranded DNA fragments are derived from the same restriction fragment;

hybridizing the first and second populations of single stranded DNA fragments to form a

Amendments to claims filed August 20, 2001
 Atty Docket No. 5525-0044.10

1. (Amended) A nucleic acid reference library derived from pooled DNA from at least two sources, said library comprising a heterogeneous mixture of nucleic acid fragments, wherein each said fragment[s comprise] (a) is a portion of a polymorphic subregion of a polymorphic consensus sequence derived from said pooled DNA or (b) is derived from a non-polymorphic subregion, [wherein]

each of said polymorphic subregions is bounded by first restriction sites and comprises an internal polymorphic restriction site which is different from said first site;

said polymorphic consensus sequence is the theoretical sequence obtained by (i) aligning said pooled DNA to provide maximum homology, and (ii) projecting each of said restriction sites onto said sequence; and

said library is enriched for fragments of type (a) relative to type (b) [other than those located between said polymorphic subregions].

2. (Amended) A nucleic acid reference library according to claim 1, wherein at least a subpopulation of said [library comprises] nucleic acid fragments further comprise[ing] oligonucleotide tags, [wherein] and different nucleic acid fragments are linked to different oligonucleotide tags.

3. (Amended) The nucleic acid reference library according to claim 2, [further comprising] wherein said fragments are contained within a replicable vector.

4. (Amended) The nucleic acid reference library according to claim 2, wherein said oligonucleotide tags comprise oligonucleotides of the form:

$$S_1S_2S_3 \dots S_n$$

wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and [wherein] said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

5. (Amended) A composition comprising subpopulations of microparticles, wherein each subpopulation comprises at least one microparticle, said microparticle comprising a polymorphic probe, wherein the polymorphic probe[s] of each [the different] subpopulation[s are] is different from those of the other subpopulations, [said others] and comprises a portion of a polymorphic subregion of a polymorphic consensus sequence as recited in claim 1.

6. (Amended) The composition of claim 5, wherein each of said subpopulations further comprises a [unique] oligonucleotide tag[s], and different subpopulations comprise different tags.

7. (Amended) The composition according to claim 6, wherein each said oligonucleotide tag[s are] is positioned between said microparticle and said polymorphic probe.

8. (Amended) The composition according to claim 6, wherein each said oligonucleotide tags comprises an oligonucleotide[s] of the form:

$$S_1 S_2 S_3 \dots S_n$$

wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and [wherein] said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

9. (Amended) An array comprising a solid support having defined regions on the surface thereof, wherein each region comprises a different polymorphic probe, and wherein each of said polymorphic probes comprises a portion of a polymorphic subregion of a polymorphic consensus sequence as recited in claim 1.

10. (Amended) The array of claim 9, wherein each of said regions further comprises an oligonucleotide tag, and different subpopulations comprise different tags.

11. (Amended) The array of claim [11] 10, wherein each said oligonucleotide tag[s are] is positioned between said surface and said polymorphic probe.

12. (Amended) The array according to claim 11, wherein each said oligonucleotide tag comprises an oligonucleotide[s] of the form:

$$S_1S_2S_3 \dots S_n$$

wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and [wherein] said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

15. (Amended) A method of making a reference library comprising a mixture of heterogeneous nucleic acid fragments, comprising:

digesting pooled nucleic acid comprising first restriction sites with a first restriction endonuclease to produce a mixture of restriction fragments;

forming a first population of single stranded nucleic DNA fragments from a first subpopulation of said restriction fragments, wherein said first subpopulation [comprises a portion of said mixture] of restriction fragments [which] comprises a second restriction site which is different from said first restriction site; [and]

forming a second population of single stranded DNA fragments from a second subpopulation of said restriction fragments, wherein said second subpopulation [comprises a portion] of said restriction fragments [that] do not contain said second restriction site, and wherein said first single stranded DNA fragments [from said first subpopulation have] are complementary [sequences] to said second single stranded DNA fragments [from said second subpopulation] when[ever] said single stranded DNA fragments are derived from the same restriction fragment;

hybridizing the first and second populations of single stranded DNA fragments to form a population of duplexes; and

isolating said duplexes to form a reference population of restriction fragments.

16. (Amended) The method of Claim [16] 15, further comprising the step of pretreating said pooled nucleic acid to enrich for non-repetitive sequences.

18. (Amended) The method of claim [18] 17, wherein said first pool of test nucleic acids is from a population of individuals having a first phenotype and said second pool of test nucleic acids is from a population of individuals having a second phenotype.

19. (New) The method of claim 17, wherein said enriching comprises selecting fragments from said pools which lack said second restriction site, and said contacting comprises contacting said selected fragments with probes which contain said second restriction site.

20. (New) The method of claim 17, wherein said enriching comprises selecting fragments from said pools which contain said second restriction site, and said contacting comprises contacting said selected fragments with probes which lack said second restriction site.

POLYMORPHIC DNA FRAGMENTS AND USES THEREOF

FIELD OF THE INVENTION

The invention relates generally to methods for isolating polymorphic DNA fragments from genomes or other nucleic acid populations, and more particularly, to a high-throughput method of isolating restriction fragments
5 containing polymorphic sequences and using such fragments for genetic identification and comparison.

BACKGROUND OF THE INVENTION

Genetic factors contribute to virtually every disease, conferring susceptibility, resistance, or influencing interaction with environmental factors, Collins *et al.*
10 (1997), *Science*, 278:1580-1581. As genome mapping and sequencing projects advance, more attention is being directed to the problem of determining sequence variability between genomes of different individuals. In the area of human health, it is believed that a detailed understanding of the correlation between genotype and disease susceptibility, responsiveness to therapy,
15 likelihood of side-effects, and other complex traits, will lead to improved therapies, improved application of existing therapies, better preventative measures, and better diagnostic procedures, Caskey (1987), *Science*, 236:1223-1229; White and Caskey (1988), *Science*, 240:1483-1488; Lander *et al.* (1994), *Science*, 265:2037-2048; Schafer *et al.* (1998), *Nature*
20 *Biotechnology*, 16:33-39; and Housman *et al.* (1998), *Nature Biotechnology*, 16:492-493.

Many techniques are available for detecting the presence or absence of suspected mutations or polymorphic sequences, including direct sequencing, ligation-based assays, restriction fragment length analysis, multiplexed and/or allele-specific polymerase chain reaction, assays based on differential electrophoretic mobilities, primer extension, mismatch repair enzymes, and specific hybridization, *e.g.*, Taylor, Editor, Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA (CRC Press, Boca Raton, 1997); Cotton, Mutation Detection (Oxford University Press, Oxford, 1997); Landegren *et al.* (1988), *Science*, 242:229-237; Landegren *et al.* (1998), *Genome Research*, 8:769-776 (1998); Brown (1994), *Current Opinion in Genetics and Development*, 4:366-373 (1994); Shumaker *et al.* (1996), *Human Mutation*, 7:346-354; Nikiforov *et al.* (1994), *Nucleic Acids Research*, 22:4167-4175; Pastinen *et al.* (1997), *Genome Research*, 7:606-614; Shuber *et al.* (1997), *Human Molecular Genetics*, 6:337-347; and the like. However, most of these techniques are not directed to large-scale identification, or surveying, of polymorphic sequences throughout whole genomes, and several of the above techniques require that the polymorphisms be known beforehand. This limitation is significant, as the frequency of single nucleotide polymorphisms in unrelated individuals has been estimated to average as high as once every several hundred basepairs, *e.g.*, Cooper *et al.* (1985), *Human Genetics*, 69:201-205; Wang *et al.* (1998), *Science*, 280:1077-1082. Thus, the number of possible sequence differences between individuals is enormous, and the task of finding significant differences, *e.g.*, those associated with disease conditions, would be extremely difficult using techniques that are applicable to only one or a few polymorphic sequences at a time.

Although several techniques have been developed for large-scale comparisons of genomes, including representational difference analysis (RDA), *e.g.*, Lisitsyn *et al.* (1993), *Science*, 259:946-951, genome mismatch scanning (GMS), *e.g.*, Nelson *et al.* (1993), *Nature Genetics*, 4:11-18, and microarray-based methods, *e.g.*, Wang *et al.* (*Id.*), and Winzeler *et al.* (1998), *Science*, 281:1194-1197,

each of these techniques has significant limitations. RDA entails repeated cycles of hybridizing highly complex mixtures of DNA and amplifying the products of such hybridizations with polymerase chain reaction (PCR). As the name of the technique indicates, the DNA involved in these operations is only a small portion of the genomes being compared (about 10%, Aldhous (1994), *Science*, 265:2008-2010) because of the difficulty of amplifying large fragments with PCR. Also, because of the complexity and size of the fragments in the hybridization reactions, it is not clear how effective the technique is in isolating subtle, yet pervasive, differences such as single nucleotide polymorphismscomplements (GMS also requires the hybridization of highly complex mixtures of DNA fragments, but more importantly the objective of the technique is to identify identical sequences in two populations; thus, it has limited applicability in analyses requiring the identification of differences, such as genetic association studies. GMS further requires the use of mismatch recognition enzymes which may have widely varying sensitivities depending on the type of enzyme employed and the type of mismatch present, *e.g.*, Cotton (*Id.*). Finally, both GMS and microarray-based methods employ arrays of DNAs complementary to the processed sequences as their primary measurement tool. Thus, the sequences suspected of being the same, in the case of GMS, or of containing polymorphisms, in the case of direct detection by microarrays, must be known before hand.

In view of the above, it would be highly desirable if there was an approach available that permitted rapid and sensitive genome-wide identification of differences in genetic composition between groups of individuals.

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SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides compositions and methods for forming nucleic acid reference libraries from

pooled genomic DNA. The reference libraries are heterogeneous mixtures enriched for polymorphic nucleic acid fragments. The polymorphic nucleic acid fragments hybridize to subregions of the pooled DNA which have a restriction site polymorphism.

- 5 The methods for making the reference libraries comprise the steps of
 - (1) digesting pooled genomic DNA with a first restriction endonuclease to form first restriction fragments; (2) forming a first population of single stranded restriction fragments from the first restriction fragments which contain a restriction site for a second restriction endonuclease; (3) forming a second
 - 10 population of single stranded restriction fragments from the first restriction fragments which lack restriction sites for the second restriction endonuclease; (4) hybridizing the first and second populations of single stranded DNA fragments to form a population of duplexes; and (5) isolating the duplexes to form a reference library. The library which is obtained is enriched for
 - 15 fragments which hybridize to genomic subregions which are polymorphic as to the restriction site for the second restriction enzyme.

The invention further provides methods for determining the ratio of such polymorphic subregions as between different populations. The methods provide a significant improvement over conventional marker associated studies,

- 20 as no sequence information is required to generate and use the reference libraries. Briefly, pooled DNA from first and second pooled test populations is digested with a first restriction endonuclease. The populations are then enriched for those fragments having a polymorphism associated with the restriction site for a second restriction endonuclease. The enriched populations
- 25 are then contacted with a reference library which is preferably created as described above using the same restriction endonucleases. Differences in the extent of hybridization provide an indication of the ratio or frequency of the different polymorphisms as between the two pools of DNA. In some

Figure 8A illustrates the reaction scheme for producing single stranded Taq⁺ fragments from Sau 3A digested pUC19 plasmids.

Figure 8B illustrates the reaction scheme for producing single stranded Taq⁻ fragments from Sau 3A digested pUC19 plasmids.

5 Figure 8C illustrates the reaction scheme for recovering double stranded Sau 3A fragments that are polymorphic with respect to Taq I.

Figures 9A and B illustrate the reaction scheme for producing single stranded Tai⁺ fragments from Bst YI digested human DNA.

10 Figures 10A and B illustrate the reaction scheme for producing single stranded Tai⁻ fragments from Bst YI digested human DNA.

Figure 11 illustrates the reaction scheme for producing a reference SNP library from Tai⁺ and Tai⁻ fragments.

DETAILED DESCRIPTION OF THE INVENTION

15 The invention is directed to reference libraries of nucleic acid fragments which are associated with nucleic acid polymorphisms. Such libraries are useful in identifying single or multiple alleles which are associated with different phenotypes. In practice the reference library is generated based upon polymorphisms within a restriction site for a restriction endonuclease.

20 The reference library which is made up of a mixture of heterogeneous nucleic acid fragments can be described by reference to Figure 1. Figure 1 depicts the relationship of various components of the invention as they relate to restriction endonuclease polymorphisms associated with one or more restriction enzymes. In Figure 1A, theoretical genomic DNA from a pool of N individuals are

aligned to provide maximum homology among the sequences. Genomic DNA from four individuals is shown in Figure 1. In Figure 1A, first endonuclease restriction sites *s* are shown which can be recognized and/or cleaved by enzyme *S*. In addition, second endonuclease restriction cleavage sites *t* are shown which are capable of being recognized and/or cleaved by restriction endonuclease *T*. The regions spanning first restriction sites *s* correspond to subregions *f*₁ through *f*₇. When genomic DNA from each of the individuals is combined as a mixture and digested with restriction endonuclease *S*, a population of restriction fragments corresponding to the subregions *f*₁ through *f*₇ is formed.

As among the sequences shown in Figure 1A, some subregions contain no *t* restriction endonuclease sites (*e.g.*, *f*₃ and *f*₅), whereas other subregions contain the *t* restriction endonuclease site in all instances, *e.g.*, *f*₆. Other subregions contain differences amongst the individuals as to whether or not the *t* restriction site is present. *See, e.g.*, *f*₁, *f*₂, *f*₄ and *f*₇. If each of these restriction sites are projected onto a single theoretical sequence, the polymorphic consensus sequence of Figure 1B is obtained. Subregions *f*₁ through *f*₇ are shown for comparative purposes. In the case of subregions *f*₁, *f*₂, *f*₄ and *f*₇, restriction site *t* is shown as being either present or absent, *i.e.*, *t*^{+/−}. Subregions *f*₁, *f*₂, *f*₄ and *f*₇ are shown in Figure 1C *vis-a-vis* their relationship to the polymorphic consensus sequence and the sequence as set forth in Figure 1A. These subregions, sometimes referred to as “polymorphic subregions”, define the reference library.

The reference library is shown in Figure 1D. As can be seen the library comprises fragments which comprise portions of the polymorphic subregions. As explained in more detail hereinafter, the method for generating this library results in enrichment for fragments other than those located between the polymorphic subregions. The library is thus skewed to have subregions *f*₁, *f*₂, *f*₄ and *f*₇ over-represented while subregions *f*₃, *f*₅ and *f*₆ are under-represented or

absent. The net effect is to decrease the complexity of the library which would otherwise be obtained by a simple double digest of the pooled genomic library with S and T. This provides a library which can be used to test other populations for polymorphisms at the t restriction site which may be associated with different phenotypes.

The reference libraries are enriched for fragments other than those located between polymorphic subregions. By "enriched" herein is meant that relative to the polymorphic subregions some or all of the fragments corresponding to non-polymorphic subregions have been selected against in the methods of the invention. Referring to Figure 1A, non-polymorphic subregions are those which contain no t restriction endonuclease site (*e.g.*, f_3 and f_5), and those which contain the t restriction endonuclease site in all instances (*e.g.*, f_6). As used herein, non-polymorphic fragments are not necessarily the same as non-polymorphic subregions.

In a preferred embodiment, 50 percent of the non-polymorphic subregions are removed. Preferably, 75 percent of the non-polymorphic subregions are removed. More preferably, 90 percent of the non-polymorphic subregions are removed, leaving a library substantially free of non-polymorphic subregions.

In a preferred embodiment, the reference library is made up of fragments of DNA corresponding to polymorphic subregions derived from a pool of individuals which is large enough so as to maximize the presence of the gene pool of a particular population. Preferably, the starting pool of nucleic acids contains 50 percent of the alleles in a given population; more preferably, 75 percent; more preferably, 90 percent; and most preferably, 95 percent.

The number of different individuals used as a source to form the nucleic acid pool from which the reference library is made determines the number of polymorphisms and alleles present in the library at given locus. For example, if

more alleles which permits the determination of haplotype associated with phenotype.

In a preferred embodiment using the reference library, a pool of DNA from individuals having a first phenotype is digested with first restriction
5 endonuclease S to form a pool of restriction fragments. Fragments which are t^- are then selected. A second pool of DNA from individuals having an second phenotype is similarly treated to select for fragments which are also t^- . The polymorphic probes are then contacted with the t^- enriched fragments and the relative frequency of the polymorphic subregions in the t^- population is
10 determined. By way of example, referring to Figure 1A, subregion f_1 is equally represented by the population of DNA from 4 individuals, half of the f_1 subregions are t^+ , the other half are t^- . Assume that this is the first population. If, by way of example only, the second population contained only $t^- f_1$ subregions, the ratio of the signal obtained in the second t^- pool would be twice
15 that obtained for the analogous pool from the first population. Such a difference would indicate that the t^- polymorphism has an association which may correlate with the observed difference in phenotype. Other associations may also be detected for one or more other polymorphic subregions.

An advantage of the present invention is that no sequence information is
20 required to generate and use the reference libraries. All that is required is the use of at least two restriction enzymes which recognize and cleave different nucleic acid sequences. In the preferred embodiments, the restriction endonuclease cleavage results in "protruding ends" with at least 4 base-pair overhangs, as opposed to blunt ends, which can be used to further manipulate
25 the restriction fragments as set forth in more detail in the methods which follow.

By "restriction site" is meant a region usually between 4 and 8 nucleotides within a nucleic acid, preferably a double stranded nucleic acid, comprising the

recognition site and/or the cleavage site of a restriction endonuclease.
 Preferably, the recognition site and cleavage site are coextensive. A
 recognition site corresponds to a sequence within a nucleic acid which a
 restriction endonuclease or group of restriction endonucleases binds to. The
 5 cleavage site corresponds to the particular point of cleavage by the restriction
 endonuclease. In the case of double stranded nucleic acid it is preferable that
 the cleavage occur at a different position on the complementary strands so as to
 provide a protruding end. Depending on the restriction endonuclease, the
 cleavage site may be within the recognition site. However, some restriction
 10 endonucleases, *e.g.*, type IIS have a cleavage site which is outside of the
 recognition site.

In a preferred embodiment, the polymorphisms which are used to generate the
 reference library are within a restriction site for a chosen enzyme. Thus, point
 mutations in the recognition and/or cleavage site can result in a restriction site
 15 which is no longer susceptible to cleavage by that particular endonuclease.
 Alternatively, the mutation can create a restriction site for an endonuclease.
 Polymorphisms such as insertions or deletions of one or more nucleotides can
 similarly result in resistance or susceptibility to digestion by a restriction
 endonuclease. Accordingly, the polymorphisms can correlate to the
 20 substitution, insertion or deletion of one or more nucleotides within a particular
 restriction site.

As used herein, the terms "mutation" and "polymorphism" are used somewhat
 interchangeably to mean a DNA molecule, such as a gene, that differs in
 nucleotide sequence from a reference DNA molecule, or wildtype, by one or
 25 more bases, insertions, and/or deletions. The usage of Cotton (*supra*) is
 followed in that a mutation is understood to be any base change whether
 pathological to an organism or not, whereas a polymorphism is usually
 understood to be a base change with no direct pathological consequences. In

moieties, *e.g.*, described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman (1990), *Chemical Reviews*, 90:543-584, or the like, with the only proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding
5 properties, reduce complexity, increase specificity, and the like.

The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides,
10 and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, *etc.*

The following provides more detailed information with regard to the preparation of the reference libraries of the invention. In a preferred embodiment, a reference population of restriction fragments is produced by the method
15 illustrated in Figures 2A through 2C. In Figure 2A, genomic DNA (200) is extracted from each of the individuals of a population of interest and pooled. By "pooled nucleic acid" herein is meant combining nucleic acid such as the genomic DNA obtained from individuals in a population of interest such that a heterogeneous mixture of nucleic acid fragments is obtained when digested with
20 at least two restriction endonucleases.

The number of individuals in the population is not critical; however, it is desirable to have the population sufficiently large so that many, if not all the polymorphic sequences of interest are captured. Preferably, the population consists of at least five individuals, and more preferably, it consists of at least
25 ten individuals. Still more preferably, the population consists of a number of individuals in the range of from 10 to 100. When the genomic DNA is combined for processing, equal amounts are preferably contributed from each genome of the population. DNA (200) is cleaved (202) with a first restriction

endonuclease S to produce a population of restriction fragments (204), to which Q adaptors are ligated (206) in a conventional ligation reaction to give fragment-adaptor complexes (208).

5 Restriction endonuclease S may be any restriction enzyme whose cleavage results in fragments with predictable protruding strands. Preferably, cleavage with first restriction enzyme S results in a protruding strand of at least four nucleotides. In further preference, restriction endonuclease S produces fragments having ends with 5' protruding strands, which allows the 3' recessed strands to be extended with a DNA polymerase in the presence of the
10 appropriate nucleoside triphosphates. In a preferred embodiment, the 3' recessed strands of such fragments are extended by one nucleotide to reduce the length of the protruding strands to three nucleotides, thereby destroying the self-complementarity of the protruding strand. This step helps to reduce self-ligation, both of the fragments and Q adaptors.

15 Q adaptors are conventional double stranded oligonucleotide adaptors which contain complementary protruding strands to those of restriction fragments (204). Q adaptors may vary widely in length and composition, but are preferably long enough to include a primer binding site for amplifying the fragment-adaptor complexes by polymerase chain reaction (PCR). Preferably,
20 the double stranded region of Q adaptors is within the range of 14 to 30 basepairs, and more preferably, within the range of 16 to 24 basepairs.

Fragment-adaptor complexes (208) are digested (210) with second restriction endonuclease, T, to produce a population (212) of fragments (213) lacking a t
25 restriction site and fragments (211) having a Q adaptor at one end and a protruding strand resulting from cleavage by T at the other end.

Restriction endonuclease T may be any restriction endonuclease different from S whose digestion of double stranded DNA leaves a protruding strand.

Preferably, T is selected so that the frequency of t restriction sites in the target DNA is significantly less than that of the s restriction sites, thereby minimizing the probability that S-produced fragments have multiple internal t restriction sites. Preferably, most S-produced fragments have no more than one potential t
5 restriction site. These conditions are satisfied by many combinations of restriction endonucleases, *e.g.*, a restriction endonuclease that has a four basepair recognition site for S and a restriction endonuclease that has a six basepair recognition site for T.

For human DNA, preferably S is a restriction endonuclease which has a four
10 nucleotide recognition site and whose cleavage results in a four nucleotide protruding strand, such as Sau 3A, Tsp 509 I, Nla III, or the like, and T is a restriction endonuclease which has a four nucleotide recognition site with a CG in its recognition sequence and whose cleavage results in at least a two
15 nucleotide protruding strand, such as Taq I, Msp I, Hin P1 I, Hha I, Aci I, or the like. Because of the "CG" deficiency in human DNA, the frequencies of the latter enzyme recognition sites are much lower than that which would be expected in random sequence DNA. For example, the Taq recognition sequence occurs at frequency of about once every 1200 basepairs, rather than about once every 256 basepairs.

20 To the mixture of fragments (212) is added M adaptors which are capable of being ligated under conventional reaction conditions to the protruding strands of fragments (211) which have an end produced by cleavage with T. Again this results in a population of at least two kinds of fragments (216): those (213) having a Q adaptor at each end ("Q-Q fragments"), and those (215a and 215b)
25 having a Q adaptor at one end and an M adaptor at the other end ("Q-M fragments"). In those instances where there are multiple t restriction sites within the same fragment "M-M fragments" are formed. If this is the case, as illustrated in Figure 8A by fragment (812), amplification with M and Q primers eliminates M-M fragments from the mixture because of a 1 base pair gap

present on one of the strands of the M-M fragments. The length of the M adaptor is selected as described for the Q adaptor; however, the sequence of the M adaptor is selected to be sufficiently different from that of the Q adaptor so that there is little or no possibility of cross-hybridization between primers during an operation such as PCR. M adaptors further have a 3' protruding strand at the end distal to the restriction fragment to which it is ligated, so that such strand is not digested by 3' exonucleases requiring double stranded DNA substrates, such as *E. coli* exonuclease III.

Alternative means for generating full-length single stranded forms of the Q-M fragment are available, including asymmetric PCR, PCR with one nuclease-resistant primer followed by exonuclease digestion, melting the complements from avidin-captured biotinylated strands, and the like, *e.g.*, Birren *et al.*, editors, *Genome Analysis: A Laboratory Manual*, Vol. 1 (Cold Spring Harbor Laboratory Press, New York, 1997); Hultman *et al.*, *Nucleic Acids Research*, 17: 4937-4946 (1989); Straus *et al.*, *BioTechniques*, 10: 376-384 (1991); Nikiforow *et al.*, *PCR Methods and Applications*, 3: 285-291 (1994); and the like, which references are incorporated by reference.

Returning to Figure 2B, mixture (216) is digested (218) with a 3' exonuclease to produce mixture (220) comprising a full length single stranded fragment (217) from each Q-M fragment (215) and two half-length single stranded fragments (219) from each Q-Q fragment (213). To mixture (220) is added (222) primer (224) specific for the primer binding site of M adaptor. After annealing, primer (224) is extended to give double stranded fragment (228), which is then amplified in a PCR using a primer specific to Q adaptor and primer (224) specific for M adaptor. Primer (224) contains several nuclease-resistant linkages at its 5' end. Preferably, the number of such linkages are in the range of from two to four. Also preferably, the nuclease resistant linkages are phosphorothioate linkages, which may be synthesized using conventional

protocols, *e.g.*, Eckstein, editor, Oligonucleotides and Analogues (IRL Press, Oxford, 1991).

5 Fragments (228) are then cleaved (232) with S to remove the Q adaptor leaving fragments (230), which are then digested with a 5' 3' exonuclease to produce a population of single stranded fragments (238). Such 5' 3' exonucleases include T7 gene 6 exonuclease (available from United States Biochemical) and may be used in accordance with the protocol of Straus *et al.*, BioTechniques 10: 376-384 (1991).

10 As shown in Figure 2C, fragments (252) from reaction mixture (204) are processed separately as follows: To fragments (252), N adaptors are ligated using conventional protocols to produce a population (256) of fragments having N adaptors at each end. The length of the N adaptor is selected as described for the Q adaptor; however, the sequence of the N adaptor is selected to be
15 sufficiently different from that of the M adaptor and Q adaptor so that there is little or no possibility of cross hybridization during an operation such as PCR. Fragments of population (256) are then cleaved (258) with T, after which the fragments of the mixture are amplified using primers specific for N; thus, the mixture is greatly enriched in fragments lacking a t restriction site. The amplified fragments are then digested (262) with a 3' exonuclease, such as *E.*
20 *coli* exonuclease III, to give a mixture (266) of single stranded half length fragments (264).

25 As shown in Figure 2D, fragments (238) and fragments (266) are combined under conditions that permit complementary strands to hybridize (268). After stable hybrids are formed, repair synthesis is performed on the hybrids to produce double stranded fragments (273), and the double stranded fragments are amplified to form the reference population of restriction fragments with respect to restriction endonucleases S and T.

The nature of the reference library will be influenced by the restriction enzymes and adaptors used to construct the library. For example, reversing the order of restriction enzymes S and T in Figures 2A-2D and adding M adaptors that bind to the s restriction site and Q and N adaptors that bind to the t restriction site will result in a reference library corresponding to polymorphisms at restriction site s. A person skilled in the art will also understand that substituting other restriction enzymes for S and T will produce fragments with different protruding ends at different sites within the nucleic acid pool. This will result in a reference library made up of fragments from different polymorphic subregions which are specifically defined by the restriction endonucleases used.

Whenever the method of the invention is applied to populations of DNA that include all or a substantial fraction of complete genomes, particularly mammalian or higher plant genomes, the step of forming hybrids may include a step of forming subpopulations of DNA in order to reduce the complexity of the DNA populations prior to hybridization. As used herein, the term "complexity" in reference to a population of polynucleotides means the number of different species of polynucleotides present in the population. For example, nucleic acid pools may be treated to reduce the complexity of DNA populations using differential PCR amplification using sets of primers having different 3'-terminal nucleotides, *e.g.*, Pardee *et al.*, U.S. Patent 5,262,311; amplification after ligation of indexing linkers, *e.g.*, Kato, U.S. Patent 5,707,807; Deugau *et al.*, U.S. Patent 5,508,169; and Sibson, U.S. Patent 5,728,524; and the like, which references are incorporated by reference. Other ways of reducing complexity include pre-treating DNA to remove repetitive sequences.

Repeated sequences are dispersed throughout eucaryotic genomes. See Davidson and Britten (1973) *The Quarterly Review of Biology*, 48:565-613; Britten and Davidson (1971) *The Quarterly Review of Biology*, 46:111-138.

In humans, repeated sequences are found at intervals of a few thousand base pairs throughout at least 80% of the genome. *See* Sealey, et al., (1985) *Nuc. Acid Res.*, 13:1905-1923. Thus, the reference library may be skewed by the presence of such repetitive elements. Such repetitive sequences may affect the polymorphic sequences present in the reference libraries because of cross hybridization which may occur between shared repetitive elements in other parts of the genome during library formation. This problem may be substantially reduced by pre-treating genomic DNA to form subpopulations of genomic DNA enriched for non-repetitive sequences.

By "repetitive sequences" herein is meant nucleotide sequences which are repeated many times and reassociate at C_0t values lower than expected from the genome size (Lin and Lee (1981) *Biochimica et Biophysica Acta*, 653:193-203).

Nucleic acid pools may be treated to form subpopulations of DNA depleted in repetitive sequences before or during the making of the reference library. Preferably 10% of the repetitive sequences are removed. More preferably 25% of the repetitive sequences are removed. Even more preferably 50% of the repetitive sequences are removed. Further reductions in repetitive sequences also may be desirable, including removal of 75% to 90% of the repetitive sequences present in the starting nucleic acid pool.

Subpopulations depleted in repetitive sequence may be formed using methods which rely on the higher effective hybridization rate of complementary nucleic acid sequences which are present at higher concentration. Thus, if a heterogeneous mixture of nucleic acid fragments is denatured and incubated under conditions that permit hybridization, those sequences present at relatively high concentrations, *e.g.*, repetitive sequences, will become double stranded more rapidly than those present at lower concentrations. The double

repetitive sequences can be separated from non-repetitive DNA by denaturing genomic DNA, allowing it to reassociate under appropriate conditions to a particular C_{ot} value, followed by separation of the double stranded molecules which bind to hydroxyapatite. See Gray et al., U.S. Patent No. 5,756,696 (issued May 26, 1998); *Current Protocols in Molecular Biology* (1997) 2.13.1-2.13.3; Soares, et al., (1994) *Proc. Natl. Acad. Sci. USA*, 91:9228-9232; Ko (1990) *Nuc. Acid Res.*, 18:5705; Kantor and Schwartz (1979) *Anal. Biochemistry*, 97:77-84.

Other approaches useful for removing repetitive DNA sequences include magnetic purification and PCR-assisted affinity chromatography (Craig, et al., (1997) *Hum. Genet.*, 100:472-476; Durm et al., (1998) *BioTechniques* 24:820-825); single stranded "absorbing" DNA attached to a solid support (Brison, et al., (1982) *Molecular and Cellular Biology*, 2:578-587; and the use of hybridization probes representative of highly repeated sequence families (Sealy, et al., (1985) *Nuc. Acids Res.*, 13:1905-1923; Wetmur (1991) *Critical Reviews in Biochemistry and Molecular Biology*, 26:227-259).

Alternatively, supopulations of nucleic acid fragments enriched for non-repetitive DNA can be formed by denaturing pooled genomic DNA and reassociating over a long period of time. This approach favors the formation of D-loops in repetitive DNA duplexes, whereas stable duplexes are formed between complementary sequences of non-repetitive DNA. Addition of single strand-specific endonucleases, such as nuclease S1, results in the removal of repetitive sequences which have formed a D-loop from the mixture, thereby enriching for non-repetitive DNA sequences. See Wetmur, (1991) *Critical Reviews in Biochemistry and Molecular Biology*, 26:227-259.

Once made, the reference libraries find use in a variety of applications. Generally, the reference libraries are used to compare the frequency of various polymorphisms in a population of interest. Polymorphisms which occur more

frequently in one population than another, can be isolated and identified using the methods of the invention. When used to analyze other populations, a pool of DNA from individuals having a first phenotype is compared to a population which demonstrates a second phenotype.

5 Accordingly, the reference libraries of the invention can be used to screen for polymorphic markers in close proximity to genes which may be associated with one or more phenotypes or genotypes. An advantage to using the reference libraries to screen for polymorphic markers associated with a phenotype or genotype is that prior knowledge of the trait is not required.

10 Thus, polymorphisms associated with genotypes which show simple Mendelian inheritance, as well as genotypes or phenotypes associated with a complex trait can be detected using the compositions and methods of the present invention. For example, response to medication, a complex trait governed by a number of genes, is amenable to this type of approach. In

15 particular, this approach can be used to identify those individuals likely to benefit from new medications being developed and those likely to suffer adverse side-effects.

Other phenotypes of biological interest which can be screened using polymorphic probes include common diseases in humans such as

20 cardiovascular diseases, autoimmune diseases, cancer, diabetes, schizophrenia, bipolar disorder and other psychiatric disorders. *See* Kwok and Gu (1999) *Mol. Medicine Today*, 5:538; Risch and Merikangas (1996) *Science*, 273:1516; Landu and Schork (1994) *Science*, 265:2037. In addition, polymorphisms in other organisms, i.e., plants, associated with phenotypical

25 traits such as disease resistance and yield can also be screened using various embodiments of the invention. *See* Kesseli *et al.* (1994) *Genetics*, 136:1435; Michelmore *et al.* (1991) *Genetics*, 88:9828.

Generally, the frequency of polymorphisms in a population of interest is compared as follows. A pool of DNA from individuals having a first phenotype is digested with a first restriction endonuclease to form a pool of restriction fragments. Fragments lacking the polymorphism are then selected.

5 A second pool of DNA from individuals having a second phenotype is similarly treated to select for subregions which also lack the polymorphism. The reference library is then contacted with the fragments which lack the polymorphism and the relative frequency of the polymorphic subregions in the individuals which lack the polymorphism is determined.

10 The pools from the two populations may be analyzed separately or mixed together and analyzed. The frequency of the polymorphism in the two populations may be determined by labeling the fragments in the two pools. The label can be the same if the two pools are analyzed separately or different labels can be used to distinguish the fragments from the two populations if the

15 pools are mixed. As explained in more detail hereinafter, labels suitable for use include light generating labels such as fluorescent dyes.

A preferred method for the use of the reference library is set forth in Figure 3. Genomic DNA is exacted from individuals of a first (300) and second (302) pool of individuals, designated X and Y, respectively, in Figure 3. Preferably,

20 equal amounts of DNA are contributed from each individual. DNA from pool X is cleaved (304) with restriction endonuclease S and B adaptors are ligated to the ends of the resulting fragments. B adaptors are selected as described above for the Q adaptors. Separately, DNA from pool Y is cleaved (306) with restriction endonuclease S and C adaptors are ligated to the ends of the

25 resulting fragments. C adaptors are selected as described above for the Q adaptors. As with the Q adaptors, the B and C adaptors contain primer binding sites for later amplification by PCR. The sequences selected for these primer binding sites should be sufficiently different that there is little or no cross hybridization of the respective primers. Equal amounts of

exonuclease III treatment, t^+ fragments are amplified using B and M primers. This selects for t^+ DNA which is then analyzed with the reference library as described above.

Once made, the reference libraries or polymorphic probes may be attached to
 5 solid phase supports either directly or via oligonucleotide tags or tag
 complements (described more fully below). Solid phase supports for use with
 the reference libraries may have a wide variety of forms, including
 microparticles, beads, membranes, slides, plates, micromachined chips, and
 the like. Likewise, solid phase supports may comprise a wide variety of
 10 compositions, including glass, plastic, silicon, alkanethiolate-derivatized gold,
 cellulose, low cross-linked and high cross-linked polystyrene, silica gel,
 polyamide, and the like.

Identical copies of the same sequence (i.e., polymorphic probes) from the
 reference library may be attached to discrete particles to form subpopulations
 15 of microparticles. A multiplicity of such subpopulations where each
 subpopulation contains different polymorphic probes forms a reference library
 composition which may be used to test other populations. Alternatively,
 identical copies of the same sequence may be attached to single or multiple
 supports such that spatially discrete regions each containing the same
 20 sequence of different polymorphic probes is formed. In the latter
 embodiment, the area of the regions may vary according to particular
 applications; usually, the regions range in area from several μm^2 , e.g., 3-5, to
 several hundred μm^2 , e.g., 100-500. Preferably, such regions are spatially
 discrete so that signals generated by events, e.g., fluorescent emissions, at
 25 adjacent regions can be resolved by the detection system being employed.

In a preferred embodiment, arrays having defined regions on the surface of
 solid phase supports can be formed using the polymorphic probes of the
 invention. Methods for creating such arrays include, but are not limited to:

Briefly, as summarized (274) in Figure 2D and illustrated more fully in Figure 4, oligonucleotide tags from a large repertoire (404) are attached (402) to the fragments (400) to form tag-fragment conjugates, a sample of tag-fragment conjugates is taken so that substantially all different fragments have different tags, the sample of tag-fragment conjugates is amplified (408), and the amplified copies (410) are specifically hybridized (414) to one or more solid phase supports (412). Preferably, the one or more solid phase supports is a population of microparticles (412) carrying oligonucleotides with complementary sequences to the tags of the tag-fragment conjugates. In the preferred embodiment employing microparticles, after specific hybridization, tag-fragments conjugates are ligated to the tag complements attached to the microparticles and the non-covalently attached strand is melt off giving microparticles (416) which are ready to accept the hybridization probes described below.

A preferred method of attaching oligonucleotide tags to fragments is further illustrated in Figures 5A and 5B. Preferably, fragments are inserted into vector (530) which after insertion comprises the following sequence of elements: first primer binding site (532), restriction site r_1 (534), oligonucleotide tag (536), junction (538), fragment (540), restriction site r_2 (542), and second primer binding site (544). After a sample is taken of the vectors containing tag-fragment conjugates the following steps are implemented: The tag-fragment conjugates are preferably amplified from vector (530) by use of biotinylated primer (548) and labeled primer (546) in a conventional polymerase chain reaction (PCR) in the presence of 5-methyldeoxycytidine triphosphate, after which the resulting amplicon is isolated by streptavidin capture. As used herein, "amplicon" means the product of an amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from a few starting sequences. Amplicons may be produced in a polymerase chain reaction or by replication in a cloning vector.

same objective, *i.e.*, rendering the tags single stranded. Such choices could include selection of different enzymes, different compositions of words making up the tags, and the like.

When the "stripping" reaction is quenched, the result is duplex (552) with
5 single stranded tag (557). After isolation, steps (558) are implemented: the tag-fragment conjugates are hybridized to tag complements attached to microparticles, a fill-in reaction is carried out to fill any gap between the complementary strand of the tag-fragment conjugate and the 5' end of tag complement (562) attached to microparticle (560), and the complementary
10 strand of the tag-fragment conjugate is covalently bonded to the 5' end (563) of tag complement (562) by treating with a ligase. This embodiment requires, of course, that the 5' end of the tag complement be phosphorylated, *e.g.*, by a kinase, such as, T4 polynucleotide kinase, or the like. The fill-in reaction is preferably carried out because the "stripping" reaction does not always halt at
15 the first G. Preferably, the fill-in reaction uses a DNA polymerase lacking 5' 3' exonuclease activity and strand displacement activity, such as T4 DNA polymerase. Also preferably, all four dNTPs are used in the fill-in reaction, in case the "stripping" extended beyond the G triplet.

As explained further below, the tag-fragment conjugates are hybridized to the
20 full repertoire of tag complements. That is, among the population of microparticles, there are microparticles having every tag sequence of the entire repertoire. Thus, the tag-fragment conjugates will hybridize to tag complements on only about one percent of the microparticles. Microparticles to which tag-fragments have been hybridized are referred to herein as "loaded
25 microparticles." For greater efficiency, loaded microparticles are preferably separated from unloaded microparticles for further processing. Such separation is conveniently accomplished by use of a FACS, or similar instrument that permits rapid manipulation and sorting of large numbers of individual microparticles. In the embodiment illustrated in Figure 6A, a

interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, *e.g.*, 3-4, to several tens of monomeric units, *e.g.*, 40-60. Whenever an oligonucleotide is represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, "T" denotes thymidine, and "U" denotes uridine, unless otherwise noted. The term "dNTP" is an abbreviation for "a deoxyribonucleoside triphosphate," and "dATP", "dCTP", "dGTP", "dTTP", and "dUTP" represent the triphosphate derivatives of the individual deoxyribonucleosides. Usually oligonucleotides comprise the natural nucleotides; however, they may also comprise non-natural nucleotide analogs. It is clear to those skilled in the art when oligonucleotides having natural or non-natural nucleotides may be employed, *e.g.*, where processing by enzymes is called for, usually oligonucleotides consisting of natural nucleotides are required.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex.

By "mismatch" herein is meant a base pair between any two of the bases A, T, (or U for RNA), G and other than the Watson-Crick base pairs G-C, and A-T. The eight possible mismatches are A-A, T-T, G-G, C-C, T-G, C-A, T-C and A-G.

5 The sequences of oligonucleotides of a minimally cross-hybridizing set differ from the sequences of every other member of the same set by at least two nucleotides. Thus, each member of such a set cannot form a duplex (or triplex) with the complement of any other member with less than two mismatches. Complements of oligonucleotide tags, referred to herein as "tag
10 complements," may comprise natural nucleotides or non-natural nucleotide analogs. When oligonucleotide tags are used for sorting, as is the case for constructing a reference DNA population, tag complements are preferably attached to solid phase supports. Oligonucleotide tags when used with their corresponding tag complements provide a means of enhancing specificity of
15 hybridization for sorting, tracking, or labeling molecules, especially polynucleotides, such as cDNAs or mRNAs derived from expressed genes.

Minimally cross-hybridizing sets of oligonucleotide tags and tag complements may be synthesized either combinatorially or individually depending on the size of the set desired and the degree to which cross-hybridization is sought to
20 be minimized (or stated another way, the degree to which specificity is sought to be enhanced). For example, a minimally cross-hybridizing set may consist of a set of individually synthesized 10-mer sequences that differ from each other by at least 4 nucleotides, such set having a maximum size of 332, when constructed as disclosed in Brenner *et al.*, International patent application
25 PCT/US96/09513. Alternatively, a minimally cross-hybridizing set of oligonucleotide tags may also be assembled combinatorially from subunits which themselves are selected from a minimally cross-hybridizing set. For example, a set of minimally cross-hybridizing 12-mers differing from one another by at least three nucleotides may be synthesized by assembling 3

subunits selected from a set of minimally cross-hybridizing 4-mers that each differ from one another by three nucleotides. Such an embodiment gives a maximally sized set of 9^3 , or 729, 12-mers.

When synthesized combinatorially, an oligonucleotide tag preferably consists of a plurality of subunits, each subunit consisting of an oligonucleotide of 3 to 9 nucleotides in length wherein each subunit is selected from the same minimally cross-hybridizing set. In such embodiments, the number of oligonucleotide tags available depends on the number of subunits per tag and on the length of the subunits.

In a preferred embodiment, the oligonucleotide tags comprise oligonucleotides of the form:

$$S_1 S_2 S_3 \dots S_n$$

As used herein, “ S_1 through S_n ” refer to the subunits comprising an oligonucleotide tag having a length from 3 to 9 nucleotides and are selected from a minimally cross hybridizing set. “ n ” is in the range from 4 to 10, and the overall length of the tag may range from 12 to 60 nucleotides.

Complements of oligonucleotide tags attached to one or more solid phase supports are used to sort polynucleotides from a mixture of polynucleotides each containing a tag. Such tag complements are synthesized on the surface of a solid phase support, such as a microparticle or a specific location on an array of synthesis locations on a single support, such that populations of identical, or substantially identical, sequences are produced in specific regions. That is, the surface of each support, in the case of a bead, or of each region, in the case of an array, is derivatized by copies of only one type of tag complement having a particular sequence. The population of such beads or regions contains a repertoire of tag complements each with distinct sequences. As used herein in reference to oligonucleotide tags and tag complements, the

term "repertoire" means the total number of different oligonucleotide tags or tag complements that are employed for solid phase cloning (sorting) or identification. A repertoire may consist of a set of minimally cross-hybridizing set of oligonucleotides that are individually synthesized, or
5 it may consist of a concatenation of oligonucleotides each selected from the same set of minimally cross-hybridizing oligonucleotides. In the latter case, the repertoire is preferably synthesized combinatorially.

Preferably, tag complements are synthesized combinatorially on microparticles, so that each microparticle has attached many copies of the
10 same tag complement. A wide variety of microparticle supports may be used with the invention, including microparticles made of controlled pore glass (CPG), highly cross-linked polystyrene, acrylic copolymers, cellulose, nylon, dextran, latex, polyacrolein, and the like, disclosed in the following exemplary references: *Meth. Enzymol.*, Section A, pages 11-147, vol. 44 (Academic
15 Press, New York, 1976); U.S. Patents 4,678,814; 4,413,070; and 4,046,720; and Pon, Chapter 19, in Agrawal, editor, *Methods in Molecular Biology*, Vol. 20, (Humana Press, Totowa, NJ, 1993). Microparticle supports further include commercially available nucleoside-derivatized CPG and polystyrene beads (*e.g.*, available from PE Applied Biosystems, Foster City, Calif.);
20 derivatized magnetic beads; polystyrene grafted with polyethylene glycol (*e.g.*, TentaGel™, Rapp Polymere, Tübingen Germany); and the like.

Microparticles may also consist of dendrimeric structures, such as disclosed by Nilsen *et al.*, U.S. Patent 5,175,270. Generally, the size and shape of a microparticle is not critical; however, microparticles in the size range of a
25 few, *e.g.*, 1-2, to several hundred, *e.g.*, 200-1000 μm diameter are preferable, as they facilitate the construction and manipulation of large repertoires of oligonucleotide tags with minimal reagent and sample usage. Preferably, glycidial methacrylate (GMA) beads available from Bangs Laboratories (Carmel, Ind.) are used as microparticles in the invention. Such
30 microparticles are useful in a variety of sizes and are available with a variety

of linkage groups for synthesizing tags and/or tag complements. More preferably, 5 μ m diameter GMA beads are employed.

- 5 Polynucleotides to be sorted, or cloned onto a solid phase support, each have an oligonucleotide tag attached, such that different polynucleotides have different tags. This condition is achieved by employing a repertoire of tags substantially greater than the population of polynucleotides and by taking a sufficiently small sample of tagged polynucleotides from the full ensemble of tagged polynucleotides. After such sampling, when the populations of supports and polynucleotides are mixed under conditions which permit
- 10 specific hybridization of the oligonucleotide tags with their respective complements, identical polynucleotides sort onto particular beads or regions. Of course, the sampled tag-polynucleotide conjugates are preferably amplified, *e.g.*, by polymerase chain reaction, cloning in a plasmid, RNA transcription, or the like, to provide sufficient material for subsequent analysis.
- 15 Oligonucleotide tags are employed for two different purposes in certain embodiments of the invention: (1) oligonucleotide tags are employed to implement solid phase cloning, as described in Brenner, U.S. Patent 5,604,097; and International patent application PCT/US96/09513, wherein large numbers of polynucleotides, *e.g.*, several thousand to several hundred
- 20 thousand, are sorted from a mixture into clonal subpopulations of identical polynucleotides on one or more solid phase supports for analysis; and (2) they are employed to deliver (or accept) labels to identify polynucleotides, such as encoded adaptors, that number in the range of a few tens to a few thousand, *e.g.*, as disclosed in Albrecht *et al.*, International patent application
- 25 PCT/US97/09472. For the former use, large numbers, or repertoires, of tags are typically required, and therefore synthesis of individual oligonucleotide tags is difficult. In these embodiments, combinatorial synthesis of the tags is preferred. On the other hand, where extremely large repertoires of tags are not required—such as for delivering labels to a plurality of kinds or

subpopulations of polynucleotides in the range of 2 to a few tens, *e.g.*, encoded adaptors, oligonucleotide tags of a minimally cross-hybridizing set may be separately synthesized, as well as synthesized combinatorially.

5 Sets containing several hundred to several thousands, or even several tens of thousands, of oligonucleotides may be synthesized directly by a variety of parallel synthesis approaches, *e.g.*, as disclosed in Frank *et al.*, U.S. Patent 4,689,405; Frank *et al.*, *Nucleic Acids Research*, 11: 4365-4377 (1983); Matson *et al.*, *Anal. Biochem.*, 224: 110-116 (1995); Fodor *et al.*, International application PCT/US93/04145; Pease *et al.*, *Proc. Natl. Acad. Sci.*, 91: 10 5022-5026 (1994); Southern *et al.*, *J. Biotechnology*, 35: 217-227 (1994), Brennan, International application PCT/US94/05896; Lashkari *et al.*, *Proc. Natl. Acad. Sci.*, 92: 7912-7915 (1995); or the like.

15 Preferably, tag complements in mixtures, whether synthesized combinatorially or individually, are selected to have similar duplex or triplex stabilities to one another so that perfectly matched hybrids have similar or substantially identical melting temperatures. This permits mis-matched tag complements to be more readily distinguished from perfectly matched tag complements in the hybridization steps, *e.g.*, by washing under stringent conditions. For combinatorially synthesized tag complements, minimally cross-hybridizing sets may be constructed from subunits that make approximately equivalent contributions to duplex stability as every other subunit in the set. Guidance for carrying out such selections is provided by published techniques for selecting optimal PCR primers and calculating duplex stabilities, *e.g.*, Rychlik *et al.*, *Nucleic Acids Research*, 17:8543-8551 (1989) and 18:6409-6412 20 (1990); Breslauer *et al.*, *Proc. Natl. Acad. Sci.*, 83: 3746-3750 (1986); Wetmur, *Crit. Rev. Biochem. Mol. Biol.*, 26: 227-259 (1991); and the like. A minimally cross-hybridizing set of oligonucleotides can be screened by additional criteria, such as GC-content, distribution of mismatches, theoretical

melting temperature, and the like, to form a subset which is also a minimally cross-hybridizing set.

The oligonucleotide tags of the invention and their complements are conveniently synthesized on an automated DNA synthesizer, *e.g.*, an Applied Biosystems, Inc. (Foster City, Calif.) Model 392 or 394 DNA/RNA Synthesizer, using standard chemistries, such as phosphoramidite chemistry, *e.g.*, disclosed in the following references: Beaucage and Iyer, *Tetrahedron*, 48: 2223-2311 (1992); Molko *et al.*, U.S. Patent 4,980,460; Koster *et al.*, U.S. Patent 4,725,677; Caruthers *et al.*, U.S. Patents 4,415,732; 4,458,066; and 4,973,679; and the like.

Oligonucleotide tags for sorting may range in length from 12 to 60 nucleotides or basepairs. Preferably, oligonucleotide tags range in length from 18 to 40 nucleotides or basepairs. More preferably, oligonucleotide tags range in length from 25 to 40 nucleotides or basepairs. In terms of preferred and more preferred numbers of subunits, these ranges may be expressed as follows:

Numbers of Subunits in Tags in Preferred Embodiments			
Monomers in Subunit	Nucleotides in Oligonucleotide Tag		
	(12-60)	(18-40)	(25-40)
3	4-20 subunits	6-13 subunits	8-13 subunits
4	3-15 subunits	4-10 subunits	6-10 subunits
5	2-12 subunits	3-8 subunits	5-8 subunits
6	2-10 subunits	3-6 subunits	4-6 subunits

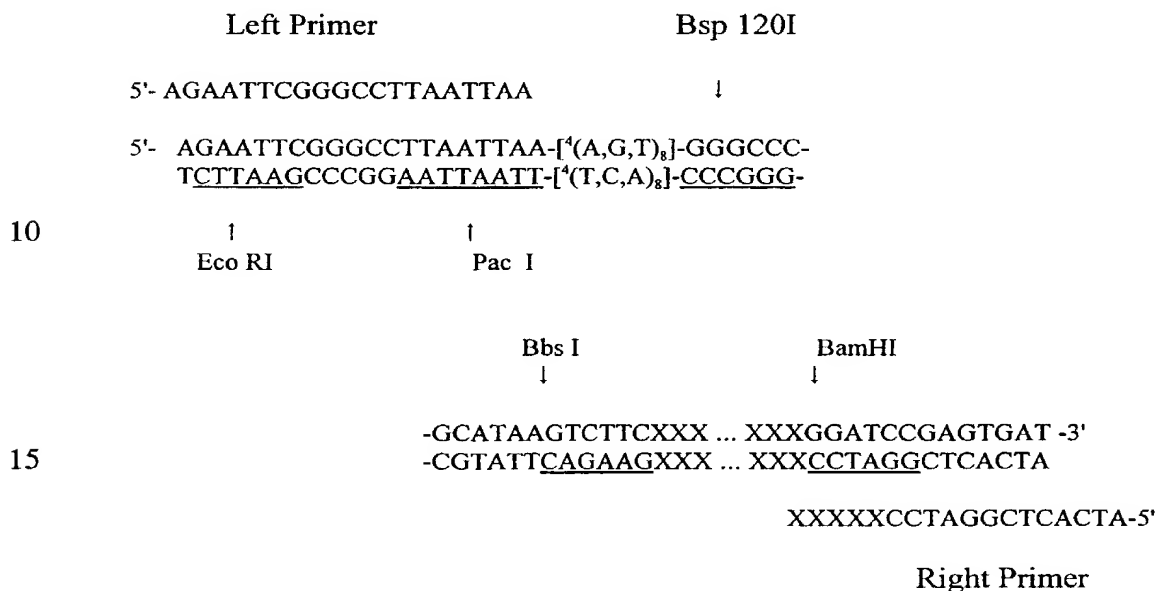
Most preferably, oligonucleotide tags for sorting are single stranded and specific hybridization occurs via Watson-Crick pairing with a tag complement.

Preferably, repertoires of single stranded oligonucleotide tags for sorting contain at least 100 members; more preferably, repertoires of such tags

contain at least 1000 members; and most preferably, repertoires of such tags contain at least 10,000 members.

Preferably, the length of single stranded tag complements for delivering labels is between 8 and 20. More preferably, the length is between 9 and 15.

5 An exemplary tag library for sorting is shown below (SEQ ID NO:1).



Formula I

20 The flanking regions of the oligonucleotide tag may be engineered to contain restriction sites, as exemplified above, for convenient insertion into and excision from cloning vectors. Optionally, the right or left primers may be synthesized with a biotin attached (using conventional reagents, *e.g.*, available from Clontech Laboratories, Palo Alto, Calif.) to facilitate purification after amplification and/or cleavage. Preferably, for making tag-fragment

25 conjugates, the above library is inserted into a conventional cloning vector, such a pUC19, or the like. Optionally, the vector containing the tag library

may contain a "stuffer" region, "XXX ... XXX," which facilitates isolation of fragments fully digested with, for example, Bam HI and Bbs I.

An important aspect of the invention is the sorting and attachment of populations of DNA sequences, *e.g.*, from a cDNA reference library, to
 5 microparticles or to separate regions on a solid phase support such that each microparticle or region has substantially only one kind of sequence attached; that is, such that the DNA sequences are present in clonal subpopulations. This objective is accomplished by insuring that substantially all different DNA sequences have different tags attached. This condition, in turn, is
 10 brought about by taking only a sample of the full ensemble of tag-DNA sequence conjugates for analysis. It is acceptable that identical DNA sequences have different tags, as it merely results in the same DNA sequence being operated on or analyzed twice. Sampling can be carried out either overtly—for example, by taking a small volume from a larger mixture—after
 15 the tags have been attached to the DNA sequences; it can be carried out inherently as a secondary effect of the techniques used to process the DNA sequences and tags; or sampling can be carried out both overtly and as an inherent part of processing steps.

If a sample of n tag-DNA sequence conjugates are randomly drawn from a
 20 reaction mixture—as could be effected by taking a sample volume, the probability of drawing conjugates having the same tag is described by the Poisson distribution, $P(r) = e^{-\lambda} (\lambda)^r / r!$, where r is the number of conjugates having the same tag and $\lambda = np$, where p is the probability of a given tag being selected. If $n = 10^6$ and $p = 1/(1.67 \times 10^7)$ (for example, if eight 4-base words
 25 described in Brenner *et al.* were employed as tags), then $\lambda = .0149$ and $P(2) = 1.13 \times 10^{-4}$. Thus, a sample of one million molecules gives rise to an expected number of doubles well within the preferred range. Such a sample is readily obtained by serial dilutions of a mixture containing tag-fragment conjugates.

As used herein, the term "substantially all" in reference to attaching tags to molecules, especially polynucleotides, is meant to reflect the statistical nature of the sampling procedure employed to obtain a population of tag-molecule conjugates essentially free of doubles. Preferably, at least ninety-five percent
5 of the DNA sequences have unique tags attached.

Preferably, DNA sequences are conjugated to oligonucleotide tags by inserting the sequences into a conventional cloning vector carrying a tag library. For example, cDNAs may be constructed having a Bsp 120 I site at their 5' ends and after digestion with Bsp 120 I and another enzyme such as
10 Sau 3A or Dpn II may be directionally inserted into a pUC19 carrying the tags of Formula I to form a tag-fragment library, which includes every possible tag-fragment pairing. A sample is taken from this library for amplification and sorting. Sampling may be accomplished by serial dilutions of the library, or by simply picking plasmid-containing bacterial hosts from colonies. After
15 amplification, the tag-fragment conjugates may be excised from the plasmid.

After the oligonucleotide tags are prepared for specific hybridization, *e.g.*, by rendering them single stranded as described above, the polynucleotides are mixed with microparticles containing the complementary sequences of the tags under conditions that favor the formation of perfectly matched duplexes
20 between the tags and their complements. There is extensive guidance in the literature for creating these conditions. Exemplary references providing such guidance include Wetmur, Critical Reviews in Biochemistry and Molecular Biology, 26: 227-259 (1991); Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory, New York,
25 1989); and the like. Preferably, the hybridization conditions are sufficiently stringent so that only perfectly matched sequences form stable duplexes. Under such conditions, the polynucleotides specifically hybridized through their tags may be ligated to the complementary sequences attached to the

microparticles. Finally, the microparticles are washed to remove polynucleotides with unligated and/or mismatched tags.

5 The specificity of hybridization of tags to their complements may be increased by taking a sufficiently small sample so that both a high percentage of tags in the sample are unique and the nearest neighbors of substantially all the tags in a sample differ by at least two words. This latter condition may be met by taking a sample that contains a number of tag-polynucleotide conjugates that is about 0.1 percent or less of the size of the repertoire being employed. For example, if tags are constructed with eight words a repertoire of 8^8 , or about 10 1.67×10^7 , tags and tag complements are produced. In a library of tag-DNA sequence conjugates as described above, a 0.1 percent sample means that about 16,700 different tags are present. If this sample were loaded directly onto a repertoire-equivalent of microparticles, or in this example a sample of 15 1.67×10^7 microparticles, then only a sparse subset of the sampled microparticles would be loaded. Preferably, loaded microparticles may be separated from unloaded microparticles by a FACS instrument using conventional protocols after DNA sequences have been fluorescently labeled and denatured. After loading and FACS sorting, the label may be cleaved prior to use or other analysis of the attached DNA sequences.

20 The following provides a more detailed explanation of how the fragments isolated in accordance with the invention are isolated and labeled using conventional techniques. A large number of light-generating labels are available for labeling fragments, including fluorescent, colorimetric, chemiluminescent, and electroluminescent labels. Generally, such labels 25 produce an optical signal which may comprise an absorption frequency, an emission frequency, an intensity, a signal lifetime, or a combination of such characteristics. Preferably, fluorescent labels are employed, either by direct incorporation of fluorescently labeled nucleoside triphosphates or by indirect application by incorporation of a capture moiety, such as biotinylated

nucleoside triphosphates or an oligonucleotide tag, followed by complexing with a moiety capable of generating a fluorescent signal, such as a streptavidin-fluorescent dye conjugate or a fluorescently labeled tag complement. Preferably, the optical signal detected from a fluorescent label is an intensity at one or more characteristic emission frequencies. Selection of fluorescent dyes and means for attaching or incorporating them into DNA strands is well known, *e.g.*, DeRisi *et al.* (cited above), Matthews *et al.*, *Anal. Biochem.*, Vol 169, pgs. 1-25 (1988); Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (Molecular Probes, Inc., Eugene, 1992); Keller and Manak, *DNA Probes*, 2nd Edition (Stockton Press, New York, 1993); and Eckstein, editor, *Oligonucleotides and Analogues: A Practical Approach* (IRL Press, Oxford, 1991); Wetmur, *Critical Reviews in Biochemistry and Molecular Biology*, 26: 227-259 (1991); Ju *et al.*, *Proc. Natl. Acad. Sci.*, 92: 4347-4351 (1995) and Ju *et al.*, *Nature Medicine*, 2: 246-249 (1996); and the like.

Preferably, light-generating labels are selected so that their respective optical signals can be related to the quantity of labeled DNA strands present and so that the optical signals generated by different light-generating labels can be compared. Measurement of the emission intensities of fluorescent labels is the preferred means of meeting this design objective. For a given selection of fluorescent dyes, relating their emission intensities to the respective quantities of labeled DNA strands requires consideration of several factors, including fluorescent emission maxima of the different dyes, quantum yields, emission bandwidths, absorption maxima, absorption bandwidths, nature of excitation light source(s), and the like. Guidance for making fluorescent intensity measurements and for relating them to quantities of analytes is available in the literature relating to chemical and molecular analysis, *e.g.*, Guilbault, editor, *Practical Fluorescence*, Second Edition (Marcel Dekker, New York, 1990); Pesce *et al.*, editors, *Fluorescence Spectroscopy* (Marcel Dekker, New York, 1971); White *et al.*, *Fluorescence Analysis: A Practical Approach* (Marcel

Dekker, New York, 1970); and the like. As used herein, the term "relative optical signal" means a ratio of signals from different light-generating labels that can be related to a ratio of differently labeled DNA strands of identical, or substantially identical, sequence that form duplexes with a complementary reference DNA strand. Preferably, a relative optical signal is a ratio of fluorescence intensities of two or more different fluorescent dyes.

Competitive hybridization between the labeled DNA strands derived from different pools of individuals is carried out by applying equal quantities of the labeled DNA strands from each such source to the microparticles loaded with the reference DNA population in a conventional hybridization reaction. The particular amounts of labeled DNA strands added to the competitive hybridization reaction vary widely depending on the embodiment of the invention. Factors influencing the selection of such amounts include the quantity of microparticles used, the type of microparticles used, the loadings of reference strands on the microparticles, reaction volume, the complexity of the populations of labeled DNA strands, and the like. Hybridization is competitive in that differently labeled DNA strands with identical, or substantially identical, sequences compete to hybridize to the same complementary reference DNA strands. The competitive hybridization conditions are selected so that the proportion of labeled DNA strands forming duplexes with complementary reference DNA strands reflects, and preferably is directly proportional to, the amount of that DNA strand in its population in comparison with the amount of the competing DNA strands of identical sequence in their respective populations. Thus, if a first and second differently labeled DNA strands with identical sequence are competing for hybridization with a complementary reference strand such that the first labeled DNA strand is at a concentration of 1 ng/l and the second labeled DNA strand is at a concentration of 2 ng/l, then at equilibrium it is expected that one third of the duplexes formed with the reference DNA would include first labeled DNA strands and two thirds of the duplexes would include second labeled

DNA strands. Guidance for selecting hybridization conditions is provided in many references, including Keller and Manak, (cited above); Wetmur, (cited above); Hames *et al.*, editors, *Nucleic Acid Hybridization: A Practical Approach* (IRL Press, Oxford, 1985); and the like.

- 5 Microparticles containing fluorescently labeled DNA strands are conveniently classified and sorted by a commercially available FACS instrument, *e.g.*, Van Dilla *et al.*, *Flow Cytometry: Instrumentation and Data Analysis* (Academic Press, New York, 1985). For fluorescently labeled DNA strands competitively hybridized to a reference strand, preferably the FACS
- 10 instrument has multiple fluorescent channel capabilities. Preferably, upon excitation with one or more high intensity light sources, such as a laser, a mercury arc lamp, or the like, each microparticle will generate fluorescent signals, usually fluorescence intensities, related to the quantity of labeled DNA strands from each cell or tissue types carried by the microparticle.
- 15 Fragments carried by microparticles may be identified after sorting, *e.g.*, by FACS, using conventional DNA sequencing protocols. Suitable templates for such sequencing may be generated in several different ways starting from the sorted microparticles carrying fragments of interest. For example, as
- 20 illustrated in Figures 6A and 6B, the reference DNA attached to an isolated microparticle may be used to generate labeled extension products by cycle sequencing, *e.g.*, as taught by Brenner, International application
- 25 PCT/US95/12678. In this embodiment, primer binding site (600) is engineered into the reference DNA (602) distal to tag complement (606), as shown in Figure 6A. After isolating a microparticle, *e.g.*, by sorting into separate microtiter well, or the like, the differentially expressed strands are melted off, primer (604) is added, and a conventional Sanger sequencing reaction is carried out so that labeled extension products are formed. These products are then separated by electrophoresis, or like techniques, for sequence determination. In a similar embodiment, sequencing templates may

be produced without sorting individual microparticles. Primer binding sites (600) and (620) may be used to generate templates by PCR using primers (604) and (622). The resulting amplicons containing the templates are then cloned into a conventional sequencing vector, such as M13. After
5 transfection, hosts are plated and individual clones are selected for sequencing.

In another embodiment, illustrated in Figure 6B, primer binding site (612) may be engineered into the competitively hybridized strands (610). This site need not have a complementary strand in the reference DNA (602). After
10 sorting, competitively hybridized strands (610) are melted off of reference DNA (602) and amplified, *e.g.*, by PCR, using primers (614) and (616), which may be labeled and/or derivatized with biotin for easier manipulation. The melted and amplified strands are then cloned into a conventional sequencing vector, such as M13, which is used to transfect a host which, in turn, is plated.
15 Individual colonies are picked for sequencing.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather
20 are presented for illustrative purposes. All references cited herein are incorporated by reference.

E X A M P L E S

Example 1

Isolation of Taq I-Polymorphic Fragments From
a Sau 3A-Digested pUC19 in the Presence
and Absence of Phage Lambda DNA

5

In this example, a conventional pUC19 plasmid was modified to create two additional Sau 3A sites between the Taq I sites located at base positions 430 and 906 of the plasmid (Figure 7A). This newly created plasmid (p0T2S) was then modified further with the addition of a Taq I site between the two new
10 Sau 3A sites, to create the plasmid p1T2S. Thus, the two plasmids are polymorphic at the new Taq I site. The two plasmids were digested separately with Sau 3A.

Single stranded portions of Sau 3A fragments containing Taq I sites (Taq⁺ fragments) were generated with the protocol outlined in Figure 8A using the
15 adaptors and primers whose sequences are listed below. The Sau 3A digested p1T2S plasmid (800) was filled in with dGTP and then an excess of Q adaptors was added (802) in a conventional ligation reaction to form product (804), which was then digested with Taq I (806) to give three possible products (808), (810), and (812). To this mixture, an excess of M adaptors
20 were added (814) in a conventional ligation reaction to form the three possible products (816), (818), and (820). Preferably, M adaptors have the following two structural features: (i) 5' extensions as shown below to prevent digestion by exonuclease III, and (ii) a protruding strand of three nucleotides at the end which is ligated to Sau 3A fragments digested by Taq I, thereby leaving a gap
25 between one strand of the adaptor and the fragment it is ligated to. This latter feature ensures that fragments with two M adaptors (*i.e.*, Taq I-Taq I fragments (820)) will not be amplified by PCR. After ligating M adaptors, the mixture was treated with exonuclease III (822) to render fragments (816) and

(818) single stranded. M and Q primers were then added to the reaction mixture and PCR was carried out (824) to form product (826) which was then digested with Sau 3A (828) to remove the Q adaptor. The resulting fragment (830) was then treated (832) with T7 gene 6 5'-exonuclease to produce the single stranded fragments (834).

Single stranded portions of Sau 3A fragments lacking Taq I sites (Taq⁻ fragments) were generated from the plasmid pOT2S with the protocol outlined in Figure 8B using the adaptors and primers whose sequences are listed below. The Sau 3A digested pOT2S was filled in with dGTP and then an excess of N adaptors were added (852) in a conventional ligation reaction to form product (854), which was then digested with Taq I (856) to give three possible products (858), (860), and (862). Preferably, the 5' ends of the N adaptors are rendered resistant to exonuclease digestion by providing phosphorothioate linkages or other protecting modifications. The reaction mixture was then treated with T7 gene 6 exonuclease to render all fragments single stranded, except those (858) having two N adaptors attached. After treatment with exonuclease I (866) to eliminate single stranded fragments, N primers were added to the reaction mixture and PCR was carried out (868) to enrich the mixture for fragment (858). The resulting fragments were then treated (860) with exonuclease III to produce the single stranded fragments (862).

As illustrated in Figure 8C, using the protocols given below, fragments (834) and (862) from the above reactions were annealed (870) and the 3' strands of the resulting duplexes (872) were extended with T4 DNA polymerase (874) to form fragments (876) having primer binding sites for M and N primers. M and N primers were added to the reaction mixture and fragments (876) were copied by PCR. The PCR amplicons from the reaction were separated by gel electrophoresis and two fragments (190 basepairs and a 230 basepairs)

were identified (lane +/- under "Plasmid") which correspond to the portions A and B of the Sau 3A fragment illustrated in Figure 7A.

The above experiment was repeated with the following alteration: an amount of phage lambda DNA equimolar to the pUC19 plasmid DNA was added to the initial Sau 3A digestion reaction. After carrying out the reactions outlined in Figures 8A-8C, the resulting fragments were separated by gel electrophoresis and bands were identified (lane +/- under "Lambda + Plasmid") which corresponded to the portions A and B of the Sau 3A fragment illustrated in Figure 7A.

The sequences for the Q, N and M adaptors are as follows:

Q adaptor: 5'-ggtacagacatggaggtgcagactaaaa
ccaugucuguaccuccacgucugauuuuucup;
N adaptor: 5'-tagtactcgtaatcagtgttcaatgta
atcatgagcattagtcacgaagttacatctap; and
M adaptor: 5'-gtctccacgtcttattctgt
tgtgagaagcagaggtgcagaataagacaagcp.

The sequences of the primers used for PCR include:

Q.top (=Q primer): 5'—ggtacagacatggaggtgcagactaaaa; N.top (=N primer): 5'—tagtactcgtaatcagtgttcaatgta; and Mn.amp (=M primer): 5'—acactcttcgtctc
cacgtcttat.

Example 2

Isolation of Tai I-Polymorphic Fragments From a BstYI-Digested Human Genomic DNA

In this example, a first sample of genomic DNA was obtained and pooled from white blood cells isolated from a population of five diabetic patients. Separately, a second sample of genomic DNA was obtained and pooled from white blood cells isolated from a population of five normal individuals. Genomic DNA from white cells was isolated from whole blood by the

protocol given below. Equal amounts of DNA from the first and second samples were combined in order to isolate Bst YI fragments ("Bst YI reference fragments") capable of containing Tai I restriction site polymorphisms. Two aliquots were removed from the combined DNA samples and were separately digested to completion with Bst YI using the manufacturer's recommended protocol. Bst YI fragments containing Tai I sites ("Tai⁺ fragments") were isolated from one aliquot by the protocol outlined in Figures 9A and 9B, and Bst YI fragments lacking Tai I sites ("Tai⁻ fragments") were isolated from the other aliquot by the protocol outlined in Figures 10A and 10B. A reference population of polymorphic fragments was then generated by combining the Tai⁺ and Tai⁻ fragments as described in Figure 11, after which the reference population may be cloned into a tag-containing vector, such as pNCV, as described below to form a library of tagged reference fragments. After transfection and expansion in an appropriate cloning vector, a sample is taken for further amplification and loading onto microparticles. Population-specific probe are then constructed as described above for identification of polymorphic sequences associated with either population.

The following is a more detailed description of the methods used to isolate Tail polymorphic fragments. First, genomic DNA is isolated and purified from Buffy-coat Preparations as follows: If the starting whole blood is 5-10 ml than you can expect approximately $10 \times 10^6 - 60 \times 10^6$ enriched leukocytes. Dilute the buffy-coat preparation at least 1/100 in phosphate-buffered saline (PBS) to determine the number of cells. There will probably be a small amount of erythrocytes in the preparation. Do not use more than 2×10^7 cells per 100/G genomic tip column (Qiagen genomic DNA kit, cat #13343). Bring the buffy-coat preparation to 5 ml with cold PBS for up to 2×10^7 cells in a 50 ml conical tube. Add 1 volume ice cold buffer lysis buffer (C1-Qiagen kit) and 3 volumes ice cold distilled water. Mix tube gently by inversion several times until the suspension is translucent. Incubate

on ice for 10 minutes. Centrifuge the lysed enriched leukocytes at 4°C for 15 minutes at 1300 x g. Discard the supernatant. Repeat wash using 1 ml C1 and 3 ml distilled water until the pellet is white (indicating that residual hemoglobin has been removed). At this point, the washed pellet can be stored

5 at -20°C without loss in yield. If continuing the protocol, resuspend the pellet in 5 ml buffer G2 (Qiagen genomic DNA kit) and vortex the nuclei at high speed for 10-30 seconds. Add 95 ul of Qiagen protease and incubate at 50°C for 30-60 minutes. The lysate should become clear at this stage. If not, extend the incubation time or pellet the undissolved material at 5000 x g

10 10 minutes 4°C. The sample should be loaded onto the Qiagen genomic tip promptly.

To purify the DNA, equilibrate a Qiagen genomic-tip 100/G with 4 ml of Buffer QBT (Qiagen kit) using gravity flow. Vortex the genomic DNA sample for 10 seconds at maximum speed and apply it to the equilibrated

15 column. Wash the Qiagen genomic tip twice with 7.5 ml Qiagen Buffer QC. Elute DNA with 5 ml of Qiagen Buffer QF. Add 3.5 ml room-temperature isopropanol and mix the tubes 10-20 times to precipitate the DNA. Dissolve the DNA pellet in water (100-200 ul) overnight on a shaker, or at 55°C for a few hours. After DNA is dissolved, dilute it 1:50 and measure the optical

20 density (OD) at 260/280. The ratio for blood cells may be low due to residual hemoglobin. The yield should be approximately 50-200 ug.

Single-stranded Tai⁺ Bst Y1 fragments are prepared by filling-in with dGTP. Ethanol precipitated BstY1 digested mixed genomic DNA is filled in with dGTP, in order to prevent concatenation of fragments in the following ligation

25 step. To fill-in with dGTP, mix: 2µl 10X Klenow buffer (500 mM Tris. HCl pH 7.5, 100 mM MgCl₂, 10 mM DTT); 500 ng BstY1 digested (ethanol precipitated) genomic DNA; 0.4µl 1.65 mM dGTP; 0.5µl 5 U/µl Klenow (Exo-); and H₂O to a final volume of 20 µl. Incubate at 37°C for 30 minutes and inactivate at 75°C for 10 minutes.

Q adaptors are ligated to both ends of the filled-in BstY1 fragments, thereby maintaining the BstY1 site. To ligate to Q adaptor, mix the following: 4 µl 5X LB1 (125 mM Tris. HCl pH 8.0, 22.5 mM DTT); 10 µl DNA; 1 µl 10 µM adaptor; 2 µl 2 mM ATP; 2.5 mM H₂O; and 0.5 µl 2000 U/µl T4 DNA ligase, in a final volume of 20 µl and incubate at 16°C overnight.

In order to produce unmethylated DNA, so that methylation-sensitive restriction enzymes (*e.g.*, Taq I) will cut to completion, the DNA is amplified using Q-top primer. Conditions for PCR are 55°C annealing temperature; 35 cycles, 30 second extension, 100 µl reaction; 0.8 µM (*i.e.*, 0.4 µM each end) primer; 2.5 mM final concentration MgCl₂; using 1 µl template (from 20 µl ligation reaction).

To purify the DNA obtained following amplification, extract with phenol/chloroform/isoamylalcohol and then with chloroform/isoamylalcohol. Precipitate with ethanol (80% ethanol wash) and resuspend in 10 µl H₂O.

The purified DNA is then digested with Tai. To digest with Tai, mix the following: 1 µg DNA; 10 µl 10X Buffer R⁺ (MBI; 100 mM Tris (pH8.5)), 100 mM MgCl₂, 1 M KCl, 1 mg/ml BSA); H₂O to 98 µl; and 2 µl Tai in a final volume of 100 µl, and incubate at 65°C for 5 hours.

Following digestion with Tai, the DNA is purified by extracting with phenol/chloroform/isoamylalcohol, followed by extraction with chloroform/isoamylalcohol. The DNA is then precipitated with ethanol (80% ethanol wash) and resuspended in 10 µl H₂O.

Next, the purified DNA is digested with Ava II. To digest with Ava II, mix the following: 10 µl 10X NEB4 (500 mM KOAc, 200 mM TrisOAc, 100 mM MgOAc, 10 mM DTT); 10 µl DNA; 2 µl Ava II (50 U/µl); and 78 µl H₂O to a final volume of 100 µl and incubate at 37°C for 5 hours.

Dephosphorylating the DNA is necessary to prevent the formation of concatomers. To dephosphorylate the DNA, mix the following: 100 μ l DNA; and 1 μ l SAP (shrimp alkaline phosphatase) (1 U/ μ l) to a final volume of 101 μ l. Incubate at 37°C for 30 minutes and inactivate at 65°C for 20 minutes.

Prior to ligating to the M adaptor, the DNA is purified. To purify the DNA, extract with phenol/chloroform/isoamylalcohol, and then extract with chloroform/isoamylalcohol. The DNA is precipitated with ethanol (80% ethanol wash) and resuspended in 10 μ l H₂O.

Ligation to the M adaptor permits BstY1 fragments to be amplified while maintaining the Tai site. The 3'-end of the M adaptor is protected from exonuclease III.

To ligate to the M adaptor, mix the following: 4 μ l 10X LB3 (250 mM Tris, pH 7.5), 25 mM MgCl₂, 25 mM DTT); 10 μ l DNA; 0.5 μ l 10 μ M M-tai adaptor; 2 μ l 2 mM ATP; 3 μ l H₂O; 0.5 μ l T4 DNA ligase (2000 U/ μ l) to a final volume of 20 μ l and incubate at 16°C overnight.

Next, the DNA is linearized with exonuclease III to produce single-stranded DNA. To treat the DNA with Exonuclease III, mix the following: 20 μ l DNA; 1 μ l ExoIII (100 U/ μ l) to a final volume of 20 μ l, and incubate at 37°C for 2 hours; then inactivate at 75°C for 10 minutes.

The DNA fragments obtained after treatment with exonuclease III are amplified using ssssMN.amp and Q-top primers. Negative controls use M primer alone and Q primer alone. To amplify the DNA, mix together the following: 39.75 μ l H₂O; 5 μ l 10X Taq buffer; 1 μ l 10mM dNTP; 1 μ l template; 1 μ l each 10 μ M primer; 2 μ l 25 mM MgCl₂ (2.5 mM final); and 0.25 μ l HS Taq to a final volume of 50 μ l. Amplify using the following

N adaptors are ligated to both ends of the filled-in Bst Y1 fragments, thereby maintaining the Bst Y1 site. 5' protected adaptors are used. To ligate to N adaptor, mix the following: 4 μ l 5X LB1 (125 mM Tris.HCl pH 8.0, 22.5 mM DTT); 10 μ l DNA; 1 μ l 10 μ M adaptor (= ssssN adaptor);
5 2 μ l 2 mM ATP; 2.5 mM H₂O; and 0.5 μ l 2000 U/ μ l T4 DNA ligase in a final volume of 20 μ l and incubate at 16°C overnight.

To produce unmethylated DNA, so that methylation-sensitive restriction enzymes (*e.g.*, Taq I) will cut to completion, the DNA obtained from the previous step is amplified using ssssN-top primer. The conditions for
10 amplification are: 50°C annealing temperature; 35 cycles, 30 second extension; 100 μ l reaction containing 0.8 μ M (*i.e.*, 0.4 μ M each end) primer, 2.5 mM final concentration MgCl₂ and template from the 20 μ l ligation reaction.

To purify the DNA following amplification, extract with
15 phenol/chloroform/isoamylalcohol, followed by extraction with chloroform/isoamylalcohol. Precipitate with ethanol (80% ethanol wash) and resuspend in 10 μ l H₂O.

The DNA purified from above is then digested with Tai. To digest with Tai, mix the following: 1 μ g DNA; 10 μ l 10X Buffer R+ (MBI; 100 mM Tris (pH8.5), 100 mM MgCl₂, 1M KCl, 1 mg/ml BSA); H₂O to 98 μ l; and 2 μ l
20 Tai1 in a final volume of 100 μ l and incubate at 65°C for 5 hours.

To prevent linear amplification of digested fragments, the DNA is first linearized with T7 gene 6 and then treated with exonuclease I. To treat the DNA with T7 gene 6, mix together the following: 100 μ l DNA; and 1 μ l
25 T7 gene 6 in a final volume of 101 μ l total.

Incubate at 23°C for 30 minutes and inactivate at 70°C for 25 minutes.

To treat the DNA with exonuclease I, mix together the following: 101 μ l DNA and 1 μ l exonuclease I in a final volume of 102 μ l, incubate at 37°C for 30 minutes and inactivate at 70°C for 25 minutes.

5 Purify the DNA by extracting first with phenol/chloroform/isoamylalcohol, and then with chloroform/isoamylalcohol. Precipitate with ethanol (80% ethanol wash) and resuspend in 10 μ l H₂O.

10 The purified DNA obtained from above is then digested with Ava II. To digest with Ava II, mix the following: 10 μ l NEB4 (500 mM KOAc, 200 mM TrisOAc, 100 mM MgOAc, 10 mM DTT); 10 μ l DNA; 79 μ l H₂O; and 1 μ l Ava II in a final volume of 100 μ l, incubate at 37°C for 5 hours and inactivate at 65°C for 20 minutes. Following digestion with Ava II, the DNA is purified by extracting first with phenol/chloroform/isoamylalcohol, followed by chloroform/isoamylalcohol, precipitating with ethanol (80% ethanol wash), and resuspending in 20 μ l H₂O.

15 Purified DNA from above is filled in with dGTP by mixing the following: 2 μ l Klenow buffer (250 mM Tris.HCl pH 7.5, 100 mM MgCl₂, 10 mM DTT); 10 μ l DNA; 0.4 μ l 1.65 mM dGTP; 0.5 μ l 5 U/ μ l Klenow (Exo-); and 7.1 μ l H₂O in a final volume of 20 μ l, incubating at 37°C for 30 minutes and inactivating at 70°C for 20 minutes.

20 Following the fill-in reaction with dGTP, the Z-adaptor is ligated onto the DNA fragments by mixing: 4 μ l 5X LB1 (250 mM Tris.HCl pH 8.0, 22.5 mM DTT); 10 μ l DNA; 1 μ l 5 μ M adaptor (=ZavaW adaptor); 2 μ l 2 mM ATP; 2.5 mM H₂O; and 0.5 μ l 2000U/ μ l T4 DNA ligase in a final volume of 20 μ l, and incubating at 16°C overnight.

After ligation of the Z-adaptors, the DNA is linearized with exonuclease III by mixing: 20 μ l DNA and 1 μ l exonuclease (100 U/ μ l) in a final volume of 21 μ l, incubating at 37°C for 2 hours and inactivating at 75°C for 10 minutes.

To amplify those fragments that lack Tai sites, mix together the following:
 5 38.75 μ l H₂O; 5 μ l 10X Taq Pol buffer; 1 μ l 10mg/ml dNTP; 1 μ l 10 μ M ssssN.top; 1 μ l 10 μ M Z.top; 2 μ l 25 mM MgCl₂; 1 μ l DNA; and 0.25 μ l HS Taq in a final volume of 50 μ l.

The DNA is then amplified under the following conditions: pretreating for 15 minutes at 95°C; followed by 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute. A final step for 5 minutes is done at 72°C. The ssssN-top primer alone as negative control. The resulting DNA is purified by extracting first with phenol/chloroform, then chloroform, precipitating with ethanol, and resuspending in 10 μ l H₂O.
 10

The final step in obtaining single-stranded Tai⁻ fragments is to linearize the DNA with T7 gene 6. This step produces full-length N-Z (Tai) fragments and is important for preventing mispriming from unrelated repetitive sequences. To treat the DNA with T7 gene 6, mix together: 8 μ l 5X T7 gene 6 buffer (200 mM Tris.HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl); 10 μ l DNA; 21 μ l H₂O; and 1 μ l T7 gene 6 in a final volume of 40 μ l. Incubate at 23°C for 60 minutes and inactivate at 75°C for 10 minutes.
 15
 20

The polymorphic Tai⁻ and Tai⁺ single stranded fragments are rescued by first hybridizing and then amplifying using N and M primers. Only those fragments containing N and M adaptors (*i.e.*, polymorphic fragments) should be amplified. Single stranded DNA samples are hybridized together by mixing the following: 4 μ l Tai⁺ DNA; 4 μ l Tai⁻ DNA; 12 μ l 1 \times Bst Y1 buffer (NEB); final volume 20 μ l. The mixture is then incubated at 94°C for 5 minutes, then cooled quickly on ice. Two μ l of 1 M NaCl is added to give a
 25

Primers used for PCR in this example are:

Q.top: ggtacagacatggagggtcagactaaaa;

SsssN.top: **tag**tactcgtaatcagtgcttcaatgta;

ssssMn.amp: acactcttcgtctccacgtcttat;

5 Mn.amp: acactcttcgtctccacgtcttat; and

Z.top: tttagaagcagactgtaagaccgtga.

Note: the nucleotides written in bold are phosphorothioates, which provide protection against T7 gene6 exonuclease (this is why the primers and adaptors have the ssss – to denote the four 5' phosphorothioate nucleotides).

10

Example 3

Construction of an Eight-Word Tag Library

An eight-word tag library with four-nucleotide words was constructed from two two-word libraries in vectors pLCV-2 and pUCSE-2. Prior to
15 construction of the eight-word tag library, 64 two-word double stranded oligonucleotides were separately inserted into pUC19 vectors and propagated. These 64 oligonucleotides consisted of every possible two-word pair made up of four-nucleotide words selected from an eight-word minimally
20 cross-hybridizing set described in Brenner, U.S. Patent 5,604,097. After the identities of the inserts were confirmed by sequencing, the inserts were amplified by PCR and equal amounts of each amplicon were combined to form the inserts of the two-word libraries in vectors, pLCV-2 and pUCSE-2. These were then used as described below to form an eight-word tag library in pUCSE, after which the eight-word insert was transferred to vector pNCV3
25 which contains additional primer binding sites and restriction sites to facilitate tagging and sorting polynucleotide fragments.

pUC19 was digested to completion with Sap I and Eco RI using the manufacturer's protocol and the large fragment isolated to give pUCSE. All restriction endonucleases, unless otherwise noted, were purchased from New England Biolabs (Beverly, Mass.). The small Sap I-Eco RI fragment was removed to eliminate the β -gal promoter sequence, which was found to skew the representation of some combinations of words in the final library. The following adaptor (SEQ ID NO:13) was ligated to the isolated large fragment in a conventional ligation reaction to give plasmid pUCSE as a ligation product.

```

10      EcoRI      Pst I      Eco RV      Hind III
          ↓          ↓          ↓          ↓
      aattctagactgcagttgatatcttaagctt
      gatctgacgtcaactatagaattcgaacga
  
```

A bacterial host was transformed by the ligation product using electroporation, after which the transformed bacteria were plated, a clone selected, and the insert of its plasmid sequenced for confirmation. pUCSE isolated from the clone was then digested with Eco RI and Hind III using the manufacturer's protocol and the large fragment was isolated. The following adaptor (SEQ ID NO:14) was ligated to the large fragment to give plasmid pUCSE-D1 which contained the first di-word (underlined).

```

          BseRI
      EcoRI  PstI      ↓      BbsI      Bsp120I      HindIII
          ↓    ↓      ↓          ↓          ↓          ↓
25      aattctgcagaggagatgaagacgaaaagaaaggggcccatgctgca
      gacgtctcctctactctgcttttctttccccgggtacgacgttcga
                                   ↑
                                   BbvI
  
```

Formula I

Further plasmids, pUCSE-D2 through pUCSE-D64, containing di-words were separately constructed from pUCSE-D1 by digesting it with Pst I and Bsp120

5'-TCG AGG CAT AAG TCT TCG AAT TCC ATC ACA CTG GGA AGA
CAA CGT AG-3'

20 Oligonucleotides S-723 and S-724 were kinased, annealed together, and
ligated to pBC.SK⁻ which had been digested with KprI and XbaI and treated
with calf intestinal alkaline phosphatase, to create plasmid pSW143.1.

Oligonucleotides S-785 and S-786 were kinased, annealed together, and ligated to plasmid pSW143.1, which had been digested with XhoI and BamHI and treated with calf intestinal alkaline phosphatase, to create plasmid pSW164.02.

5 Oligonucleotides S-960, S-961, S-962, and S-963 were kinased and annealed together to form a duplex consisting of the four oligonucleotides. Plasmid pSW164.02 was digested with XhoI and SapI. The digested DNA was electrophoresed in an agarose gel, and the approximately 3045 bp product was purified from the appropriate gel slice. Plasmid pUC4K (from Pharmacia)
10 was digested with PstI and electrophoresed in an agarose gel. The approximately 1240 bp product was purified from the appropriate gel slice. The two plasmid products (from pSW164.02 and pUC4K) were ligated together with the (S-960/961/962/963) duplex to create plasmid pLCVa.

DNA from Adenovirus5 (New England Biolabs) was digested with PacI and
15 Bsp120I, treated with calf intestinal alkaline phosphatase, and electrophoresed in an agarose gel. The approximately 2853 bp product was purified from the appropriate gel slice. This fragment was ligated to plasmid pLCVa which had been digested with PacI and Bsp120I, to create plasmid pSW208.14.

Plasmid pSW208.14 was digested with XhoI, treated with calf intestinal
20 alkaline phosphatase, and electrophoresed in an agarose gel. The approximately 5374 bp product was purified from the appropriate gel slice. This fragment was ligated to oligonucleotides S-1105 and S-1106 (which had been kinased and annealed together) to produce plasmid pLCVb, which was then digested with Eco RI and Hind III. The large fragment was isolated and
25 ligated to the Formula I adaptor (SEQ ID NO:14) to give pLCV-D1.

As above for pUCSE, further plasmids, pLCV-D2 through pLCV-D64, containing di-words were separately constructed from pLCV-D1 by digesting

it with Pst I and BspI20 I, isolating the large fragment, and a ligating an adaptor of Formula II. After cloning and isolation, the inserts of the vectors were sequenced to confirm the identities of the di-words.

Each of the vectors pLCV-D1 through -D64 and pUCSE-D1 through -D64
5 was separately amplified by PCR. The components of the reaction mixture were as follows:

10 10 µl template (about 1-5 ng)
 10 µl 10x Klentaq™ buffer (Clontech Laboratories, Palo Alto, Calif.)
 2.5 µl biotinylated DF primer at 100 pmoles/l
 2.5 µl biotinylated DR primer at 100 pmoles/l
 2.5 µl 10 mM deoxynucleoside triphosphates
 5 µl DMSO
 66.5 µl H₂O
 1 µl Advantage Klentaq™ (Clontech Laboratories, Palo Alto, Calif.)

15 The temperature of the reactions was controlled as follows: 94°C for 3 minutes; 25 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 10 seconds; followed by 72°C for 3 minutes, then 4°C. The DF and DR primer binding sites were upstream and downstream portions of the vectors selected to give amplicons of 104 basepairs in length. After the reactions
20 were completed, 5 µl of each PCR product were separated polyacrylamide gel electrophoresis (20% with 1xTBE) to confirm by visual inspection that the reaction yields were approximately the same for each PCR. After such confirmation, using conventional protocols, 10 µl of each PCR was extracted twice with phenol and once with chloroform, after which the DNA in the
25 aqueous phase was precipitate with ethanol. After resuspension in 200 µl of 1x NEB buffer #2 (New England Biolabs, Beverly, Mass.), the DNA was cleaved with Bbv I and Eco RI by adding the enzymes in 50 µl of the manufacturer's reco mMended buffer. The digestion resulted in the production of three fragments: a biotinylated fragment of 38 basepairs, a
30 di-word-containing fragment of 29 basepairs, and a biotinylated fragment of 37 basepairs. After completion of the reaction, the excess biotinylated

primers were removed by adding 50 µl 50% Ultralink (streptavidin-Sepharose, Pierce Chemical Co., Rockford, Ill.) and vortexing the mixture at room temperature for 30 minutes. The Ultralink material was separated from the reaction mixture by centrifugation, after which approximately half of the mixture was separated by polyacrylamide gel electrophoresis (20% gel). The 29-basepair band was cut out of the gel and the 29-basepair fragment was eluted using the "crush and soak" method, *e.g.*, Sambrook *et al.*, *Molecular Cloning*, Second Edition (Cold Spring Harbor Laboratory, New York, 1989). This material was then ligated into either pLCV-D1 or pUCSE-D1 after the latter were digested with Bbs I and Eco RI and treated with calf intestinal alkaline phosphatase, using the manufacturer's reco mMend protocols.

pNCV3 was constructed by first assembling the following fragment (SEQ ID NO:26) from synthetic oligonucleotides:

```

15      EcoRI
        |
aattctgtaaaacgacggccagtcgccagggttttcccagtcacgacgtgaataaatag-
gacattttgctgcccggtcagcgggtccaaaagggtcagtgctgcacttatttatc-

20      PacI                               Bsp120I
        |                               |
ttaattaaggaataggcctctcctcgagctcggtaccgggcccgcataagtcttc-
aattaattccttatccggagaggagctcgagccatggcccggcggtattcagaag-

25      ClaI           EcoRV       SapI       BamHI
        |             |           |           |
atctatcgatgattgaagagcgatatcgctcttcaatcgatccatcc-
tagatagctactaacttctcgctatagcgagaagttagcctaggtagg-
        |
        SapI

30                               HindIII
                               |
tcaactaattaccacacaacatacgagccggaagcgggtcatagctgtttcctga
agttgattaatggtgtgtgtgtatgctcgcccttcgccagtatcgacaaaggacttcga

```

After isolation, the fragment was cloned into Eco RI and Hind III-digested pLCV-D1 using conventional protocols.

The di-words of pLCV-2 were amplified either by PCR or plasmid expansion, the product was digested with Eco RI and BbvI after which the Eco RI-BbvI fragment was isolated as insert 1. Two-word library pUCSE-2 was digested with Eco RI, Bbs I, and Pst I, after which the large fragment was treated with calf intestinal alkaline phosphatase to give vector 1. Vector 1 and insert 1 were combined in a conventional ligation reaction to give three-word library, pUCSE-3. pUCSE-3 was digested with Eco RI, Bbs I, and Pst I, after which the large fragment was treated with calf intestinal alkaline phosphatase to give vector 2. Vector 2 and insert 1 were then combined in a conventional ligation reaction to give four-word library, pUCSE-4. The 4-mer words of pUCSE-4 were amplified either by PCR or plasmid expansion, the product was digested with Eco RI and BbvI after which the Eco RI-BbvI fragment was isolated as insert 2. pLCV-2 was digested with Eco RI, Bbs I, and Pst I, after which the large fragment was treated with calf intestinal alkaline phosphatase to give vector 3. Vector 3 and insert 2 were then combined in a conventional ligation reaction to give five-word library, pLCV-5. The 5-mer words of pLCV-5 were amplified either by PCR or plasmid expansion, the product was digested with Eco RI and BbvI after which the Eco RI-BbvI fragment was isolated as insert 3. pUCSE-4 was digested with Eco RI, Bbs I, and Pst I, after which the large fragment was treated with calf intestinal alkaline phosphatase to give vector 4. Vector 4 and insert 3 were then combined in a conventional ligation reaction to give eight-word library, pUCSE-8. The 8-mer words of pUCSE-8 were amplified either by PCR or plasmid expansion, the product was digested with Bse RI and Bsp120 I, after which the BseRI-Bsp120I fragment was isolated as insert 4. pNCV3 was digested with Bse RI, Bsp120 I, and Sac I, after which the large fragment was isolated and treated with calf intestinal alkaline phosphatase to give vector 5. Vector 5 was then combined with insert 4 in a conventional ligation reaction to give the eight-word library pNCV3-8.

CLAIMS

What is claimed is:

1. A nucleic acid reference library comprising a heterogeneous mixture of nucleic acid fragments, wherein said fragments comprises a portion of a polymorphic subregion of a polymorphic consensus sequence, wherein each of said polymorphic subregions is bounded by first restriction sites and comprises an internal polymorphic restriction site which is different from said first site and wherein said library is enriched for fragments other than those located between said polymorphic subregions.
2. A nucleic acid reference library according to claim 1 wherein at least a subpopulation of said library comprises nucleic acid fragments further comprising oligonucleotide tags wherein different nucleic acid fragments are linked to different oligonucleotide tags.
3. The nucleic acid reference library according to claim 2 further comprising a replicable vector.
4. The nucleic acid reference library according to claim 2 wherein said oligonucleotide tags comprise oligonucleotides of the form:
$$S_1 S_2 S_3 \dots S_n$$
wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally cross-hybridizing set, n is in the range of from 4 to 10, and wherein said tag has a length in the range of from 12 to 60 nucleotides or base pairs.
5. A composition comprising subpopulations of microparticles wherein each subpopulation comprises at least one microparticle comprising a polymorphic probe wherein the polymorphic probes of the different subpopulations are different from said others and comprise a portion of a polymorphic subregion of a polymorphic consensus sequence.

6. The composition of claim 5 wherein each of said subpopulations further comprises a unique oligonucleotide tags.
7. The composition according to claim 6 wherein said oligonucleotide tags are positioned between said microparticle and said polymorphic probe.
- 5 8. The composition according to claim 6 wherein said oligonucleotide tags comprise oligonucleotides of the form:
$$S_1S_2S_3 \dots S_n$$
wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally
10 cross-hybridizing set, n is in the range of from 4 to 10, and wherein said tag has a length in the range of from 12 to 60 nucleotides or base pairs.
9. An array comprising a solid support having defined regions on the surface thereof, wherein each region comprises a different polymorphic probe, and wherein each of said polymorphic probes comprises a portion of a
15 polymorphic subregion of a polymorphic consensus sequence.
10. The array of claim 9 wherein each of said regions further comprises an oligonucleotide tag.
11. The array of claim 11 wherein said oligonucleotide tags are positioned between said surface and said polymorphic probe.
- 20 12. The array according to claim 11 wherein said oligonucleotide tag comprises oligonucleotides of the form:
$$S_1S_2S_3 \dots S_n$$
wherein each of S_1 through S_n are subunits consisting of an oligonucleotide having a length from 3 to 9 nucleotides and are selected from a minimally

cross-hybridizing set, n is in the range of from 4 to 10, and wherein said tag has a length in the range of from 12 to 60 nucleotides or base pairs.

13. A nucleic acid reference library of genomic DNA from a plurality of individuals comprising:

5 a heterogeneous mixture of restriction fragments of a first restriction endonuclease;

wherein said restriction fragments from the same locus contain members having one or more restriction site polymorphisms with respect to a second restriction endonuclease, the number of such members forming a proper subset of the total number of restriction fragments from said locus.

14. A nucleic acid reference library of genomic DNA from a plurality of individuals comprising:

a heterogeneous mixture of restriction fragments of a first restriction endonuclease;

15 wherein said restriction fragments from the same locus contain at least one member having one or more restriction site polymorphisms with respect to a second restriction endonuclease and at least one member without said restriction site polymorphism.

15. A method of making a reference library comprising a mixture of heterogeneous nucleic acid fragments comprising:

20 digesting pooled nucleic acid comprising first restriction sites with a first restriction endonuclease to produce a mixture of restriction fragments;

forming a first population of single stranded nucleic DNA fragments from a first subpopulation of said restriction fragments, wherein said first subpopulation comprises a portion of said mixture of restriction fragments which comprises a second restriction site which is different from said first restriction site; and

forming a second population of single stranded DNA fragments from a second subpopulation of said restriction fragments wherein said second subpopulation comprises a portion of said restriction fragments that do not contain said second restriction site, and wherein said first single stranded DNA fragments from said first subpopulation have complementary sequences to said second single stranded DNA fragments from said second subpopulation whenever said single stranded DNA fragments are derived from the same restriction fragment;

hybridizing the first and second populations of single stranded DNA fragments to form a population of duplexes; and

isolating said duplexes to form a reference population of restriction fragments.

16. The method of Claim 16 further comprising the step of pretreating said pooled nucleic acid to enrich for non-repetitive sequences.

17. A method for determining the ratio of a polymorphic subregion between at least two different pools of test nucleic acid comprising generating a first pool of restriction endonuclease fragments from a first pool of test nucleic acids comprising first restriction sites by digesting said pool with a first restriction endonuclease;

generating a second pool of restriction endonuclease fragments from a second pool of test nucleic acids comprising first restriction sites by digesting said pool with said first restriction endonuclease;

enriching said first and said second pools of restriction fragments for those fragments which contain a polymorphism associated with a second restriction site to form first and second enriched populations;

contacting said first and said second enriched populations with a reference library comprising probes enriched for subregions which are polymorphic for said second restriction site; and

determining the ratio of binding of said probes with said first and said second enriched populations.

18. The method of claim 18 wherein said first pool of test nucleic acids is from a population of individuals having a first phenotype and said second pool of test nucleic acids is from a population of individuals having a second phenotype.

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(54) Title: POLYMORPHIC DNA FRAGMENTS AND USES THEREOF

(57) Abstract: The invention provides methods and materials for generating a reference library of restriction fragments from pooled nucleic acids that contain a sequence polymorphism. Preferably, such a library is formed by digesting genomic DNA from a pool of individuals with a first and a second restriction endonuclease to form a population of restriction fragments; isolating restriction fragments of the population digested by both the first and second restriction endonucleases and forming a first single stranded fragment population therefrom; separately isolating restriction fragments from the population digested by the first restriction endonuclease but not the second restriction endonuclease and forming a second single stranded fragment population therefrom; hybridizing the first and second single stranded fragment populations to form a population of duplexes; and isolating the population of duplexes to form a reference library of restriction fragments that contain sequence polymorphism. An important aspect of the invention is the use of the reference population of restriction fragments to compare the frequencies of polymorphic sequences between different population pools. Such comparisons may be accomplished by competitively hybridizing DNA from the respective pools which has been enriched for the presence of a restriction site polymorphism with DNA from the reference population. Preferably, such competitive hybridization reactions are carried out the reference library attached to one or more solid phase supports. Most preferably, members of the reference library are attached to individual microparticles so that each microparticle has a unique fragment attached. After competitive hybridization, the microparticles may be analyzed and sorted for identifying those microparticles carrying sequences for which the pools being compared exhibit different polymorphic frequencies.

WO 00/50632 A3

FIGURE 1A

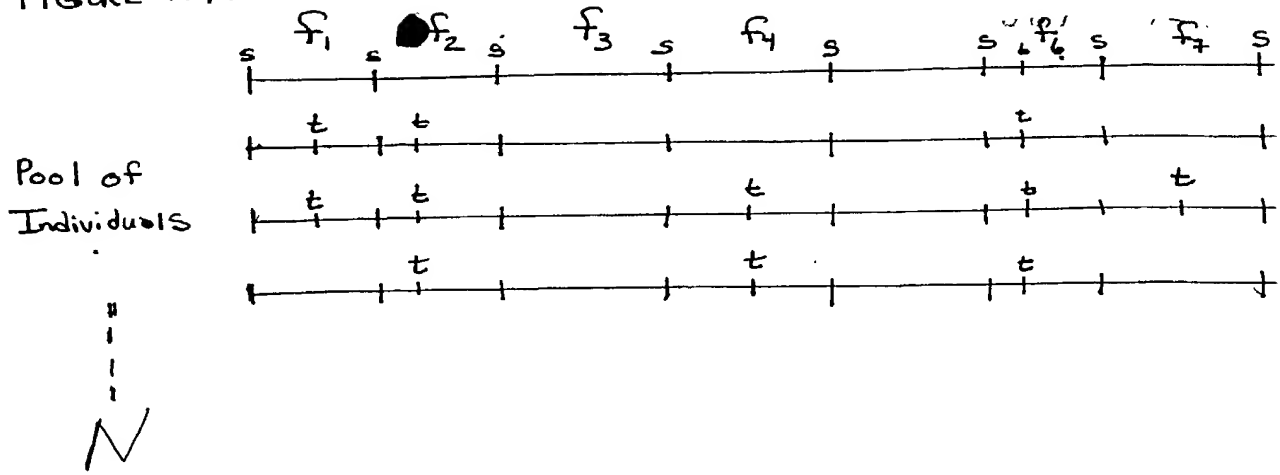


FIGURE 1B

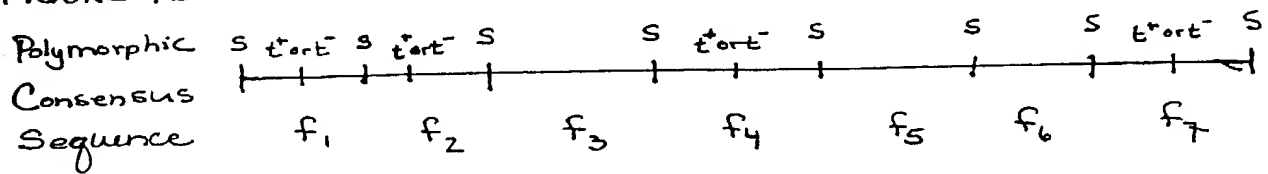


FIGURE 1C

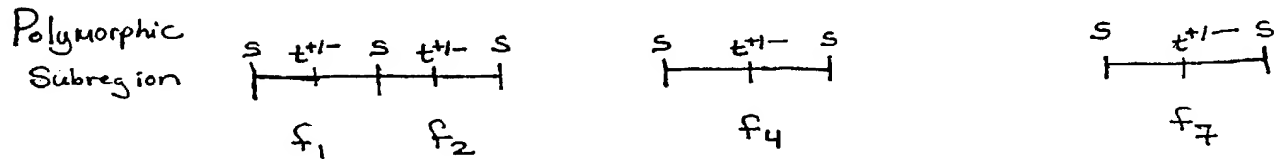
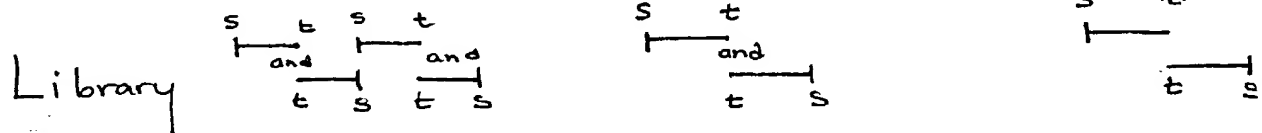


FIGURE 1D



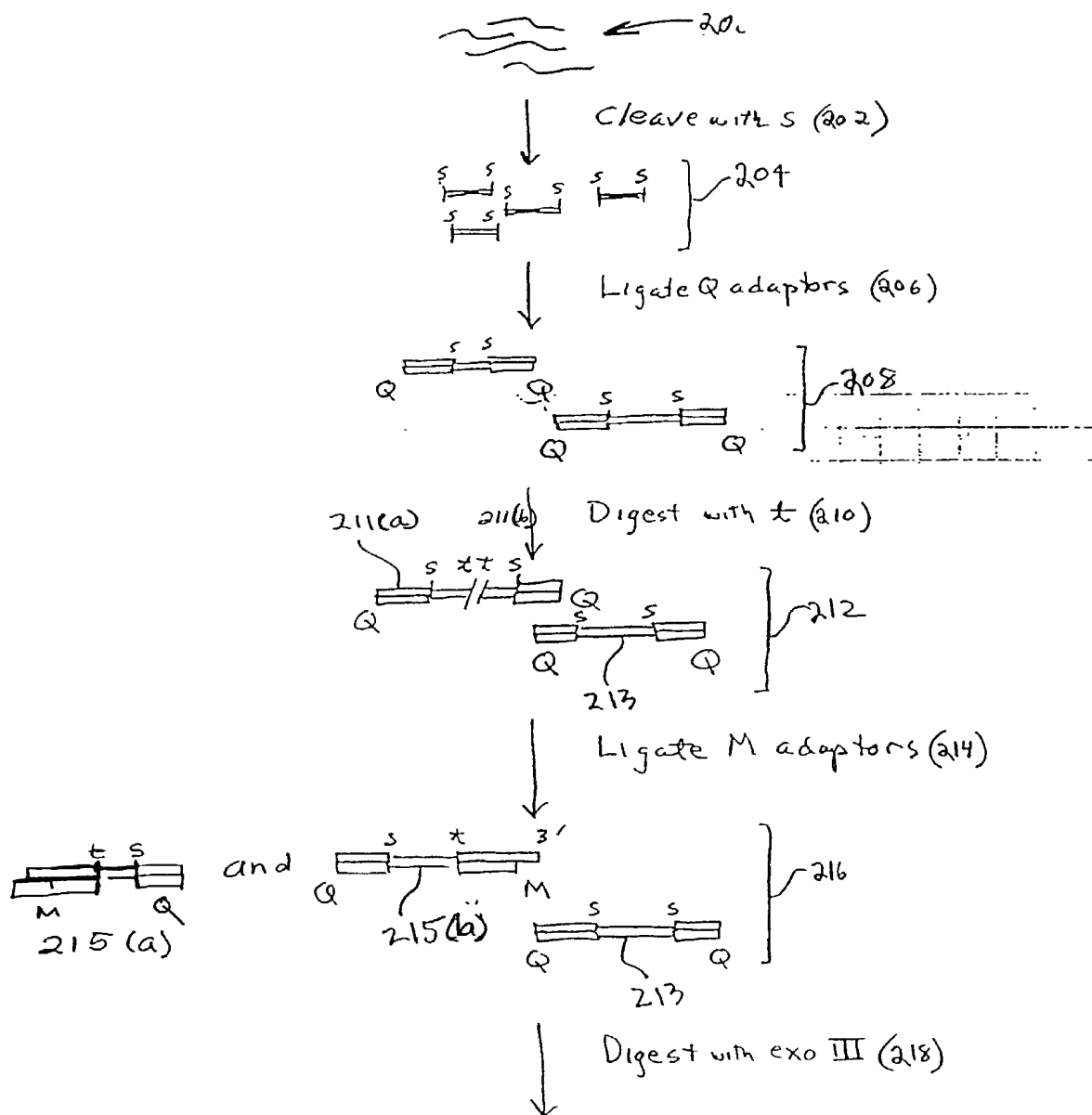


Fig. 2A

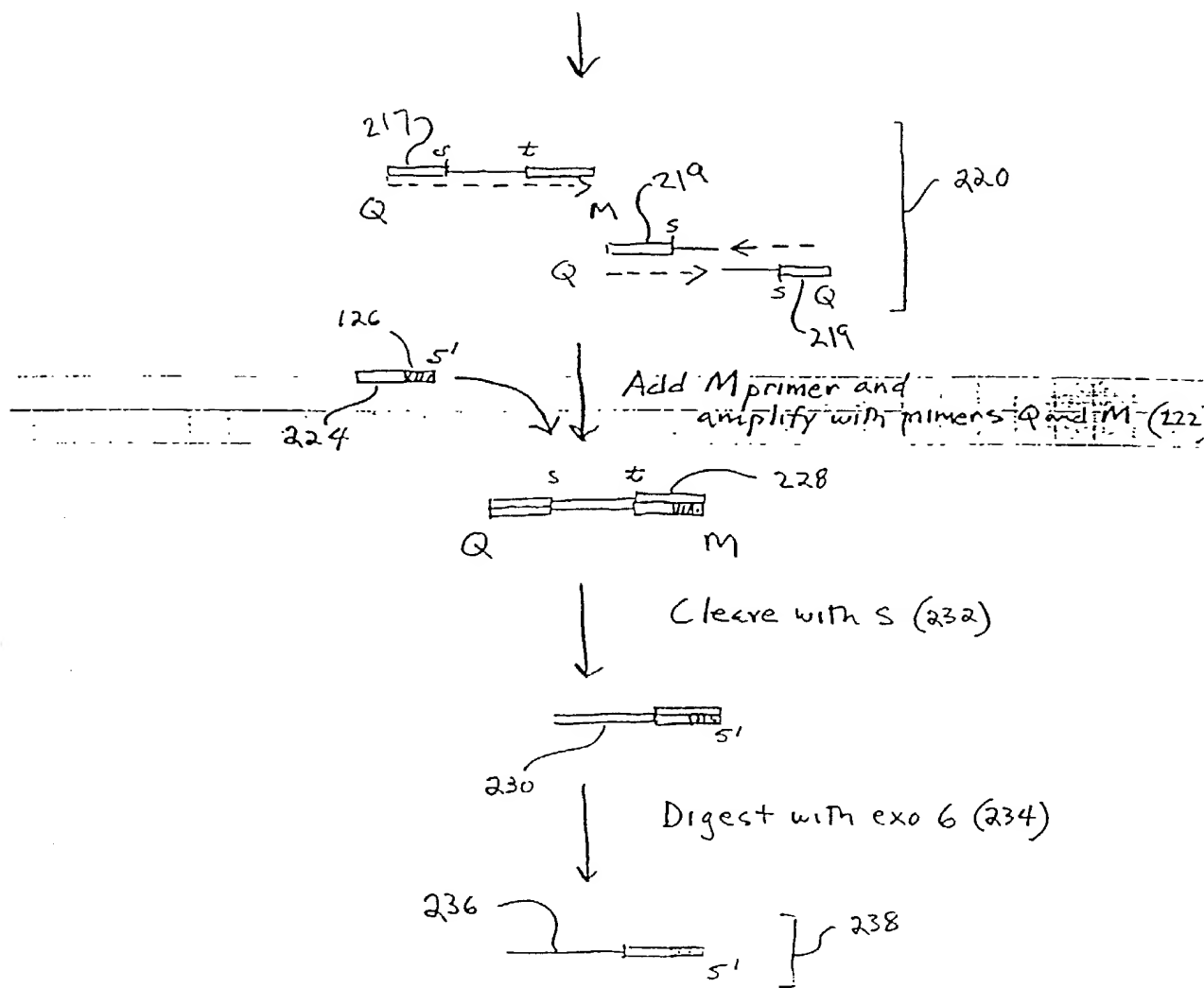


Fig 2B

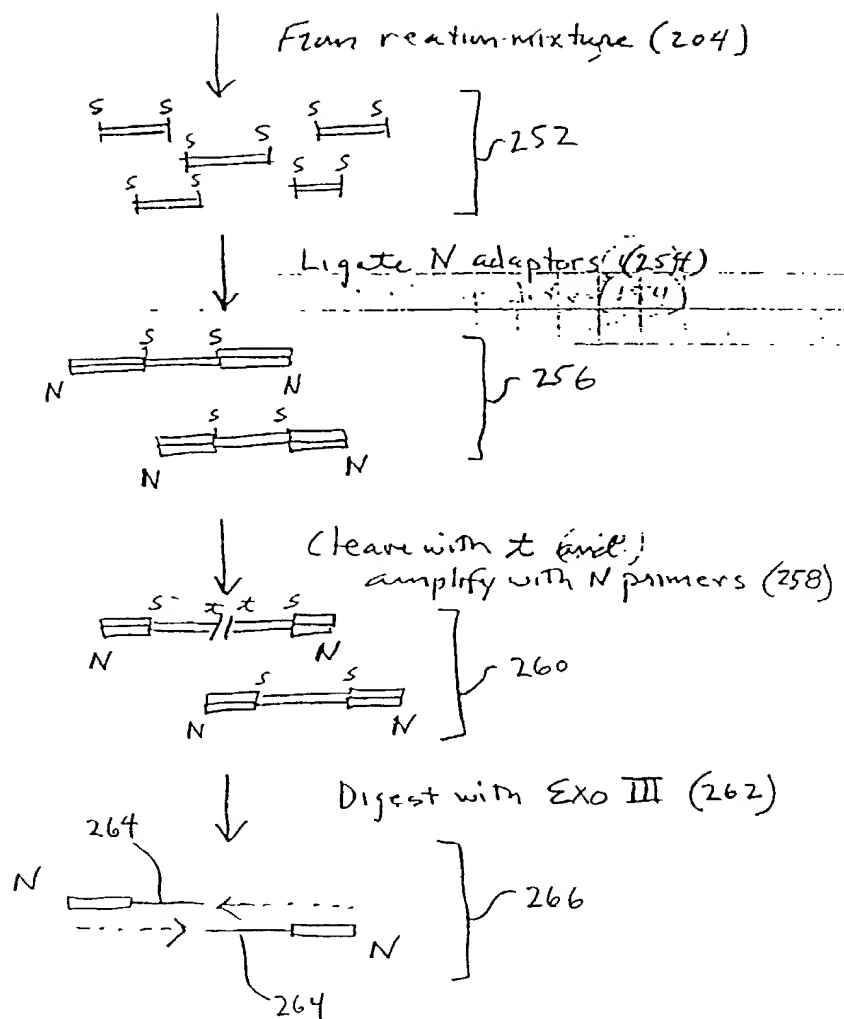


Fig. 2C

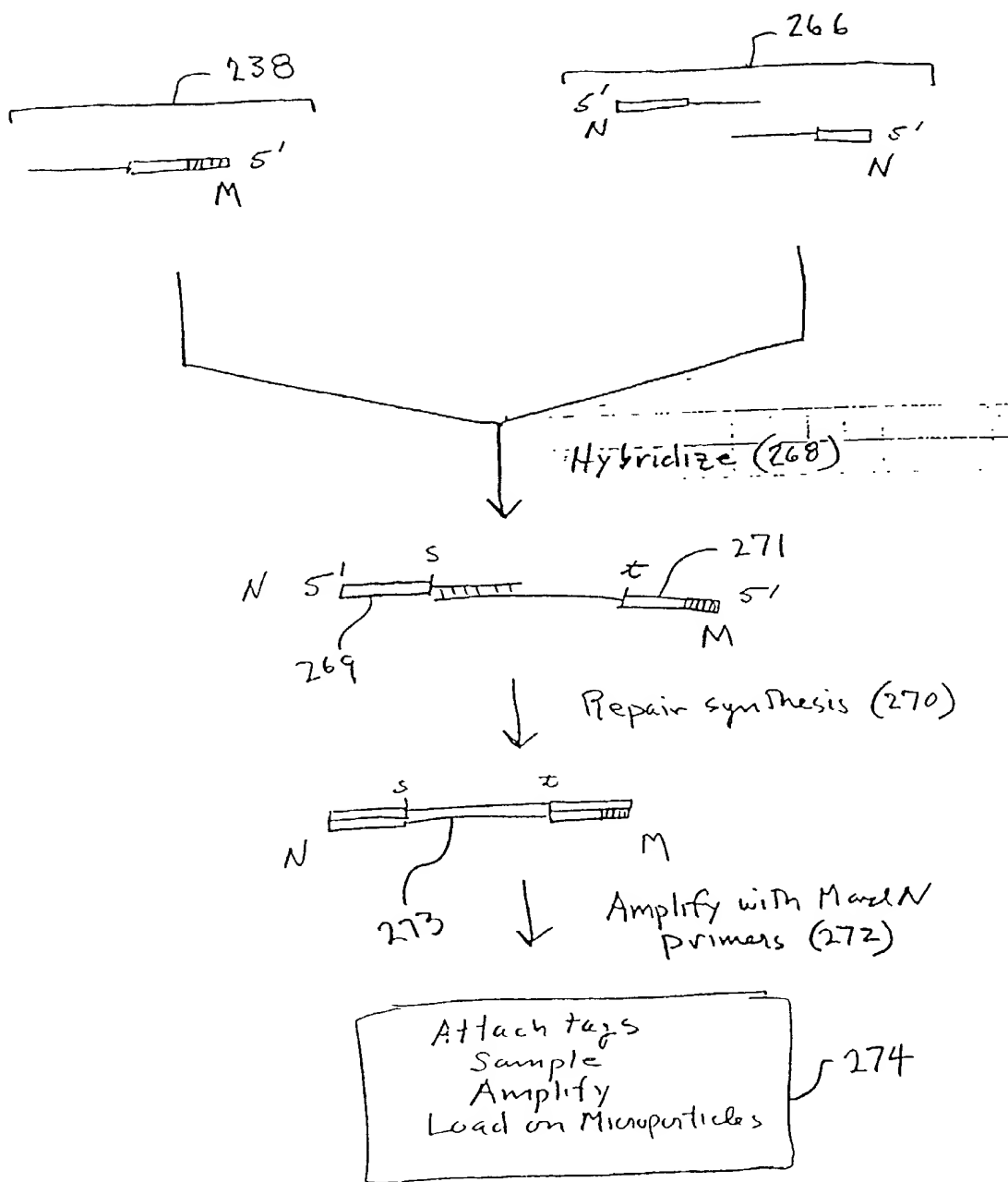
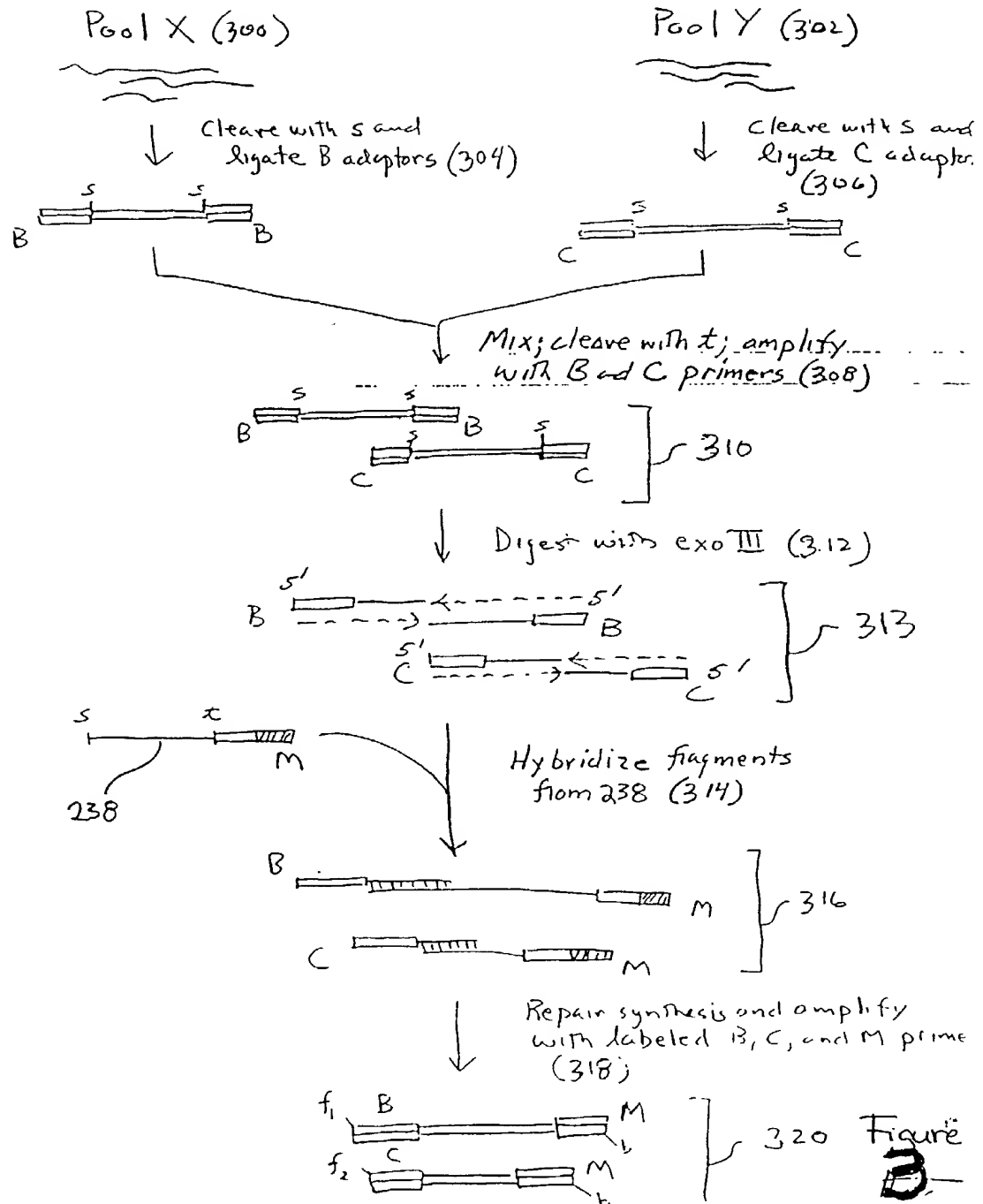


Fig. 21



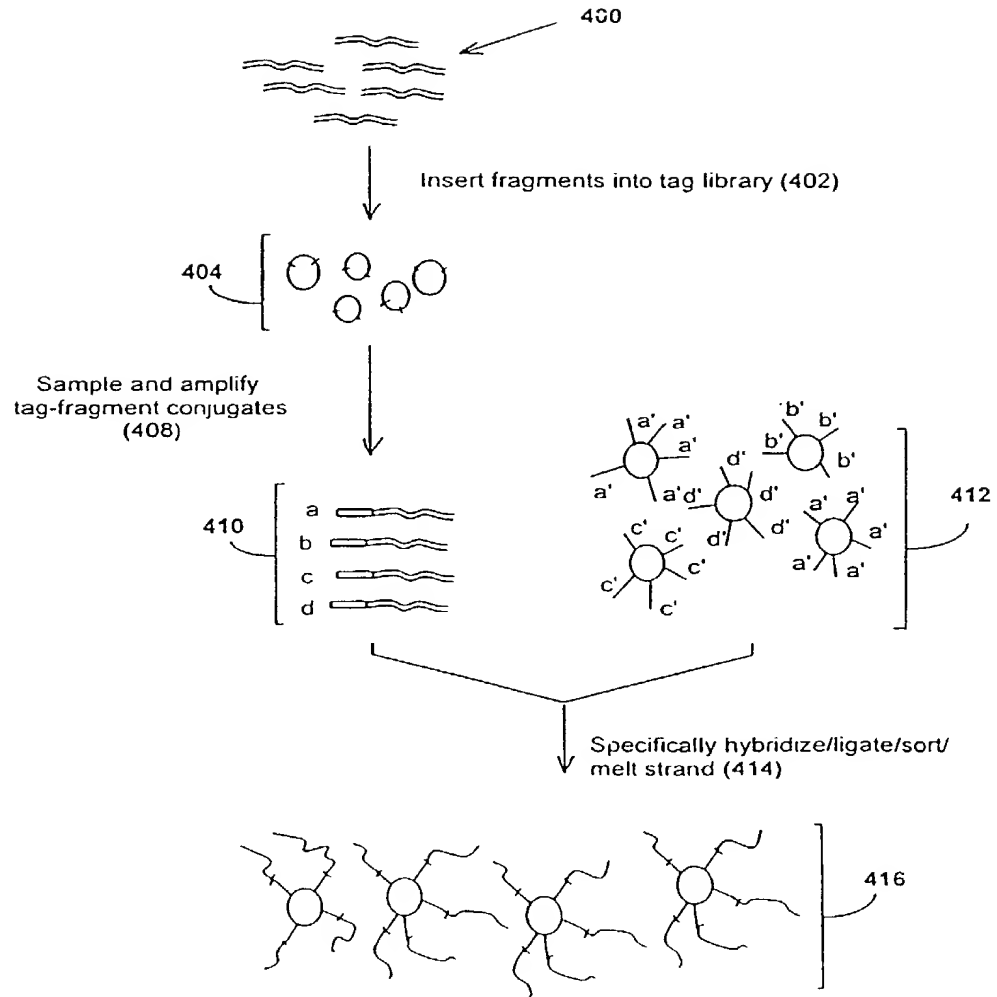


Fig. 4

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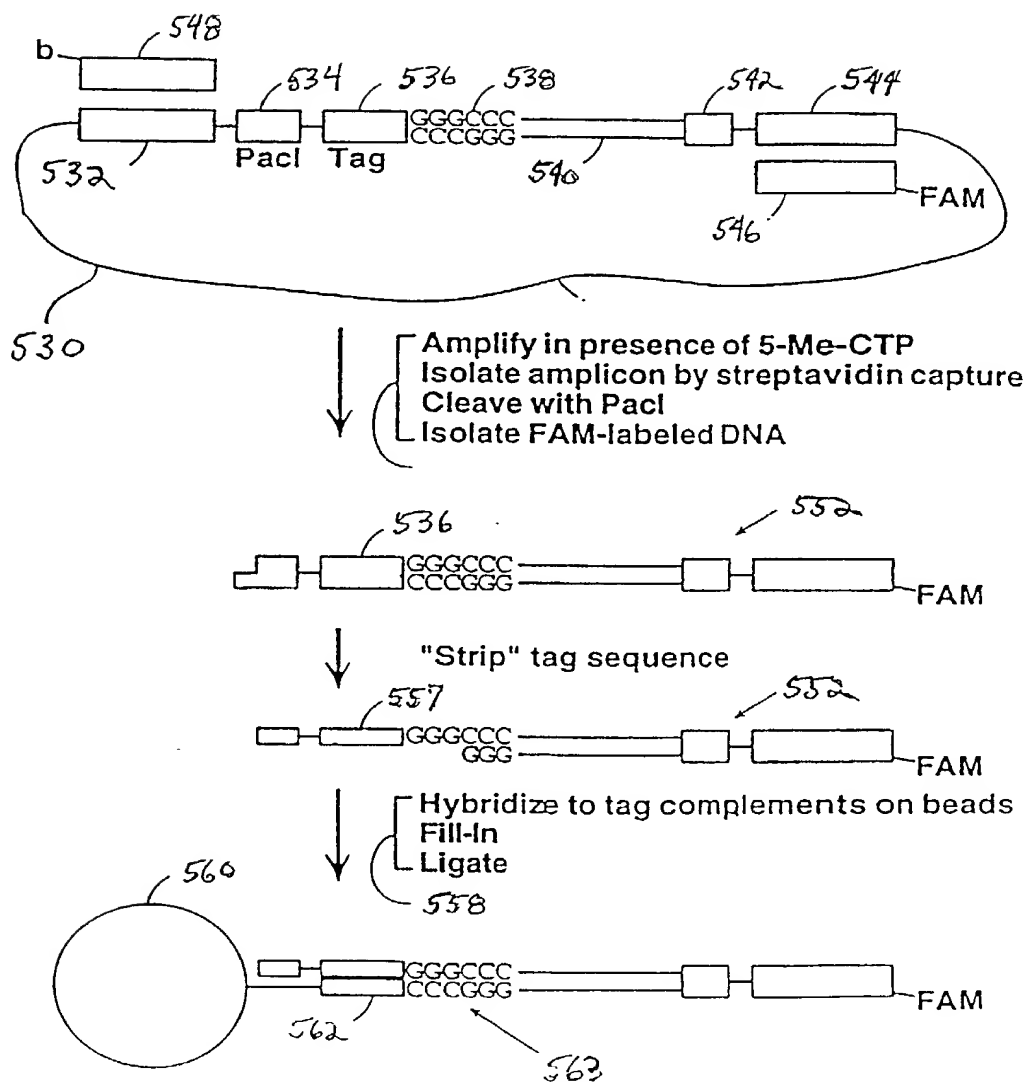


Fig 5A

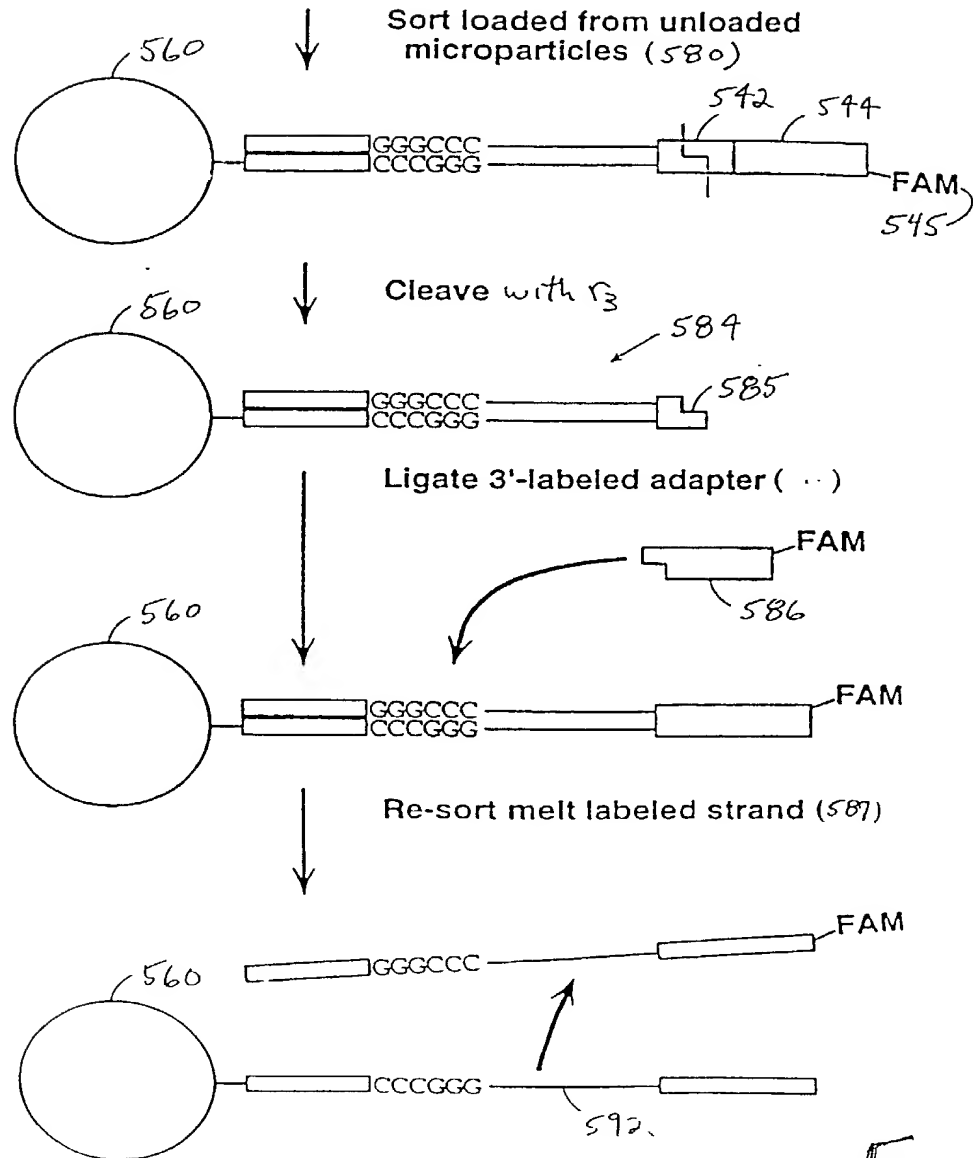


FIG. 5
B

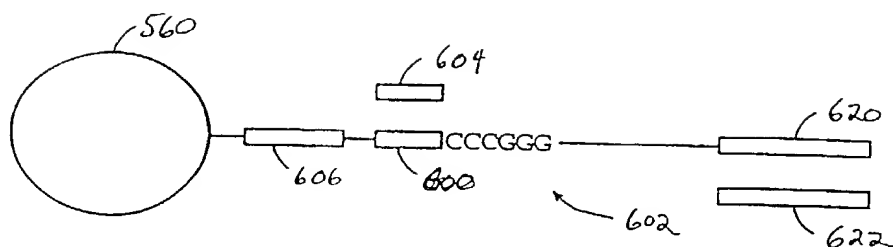


Fig. 6A

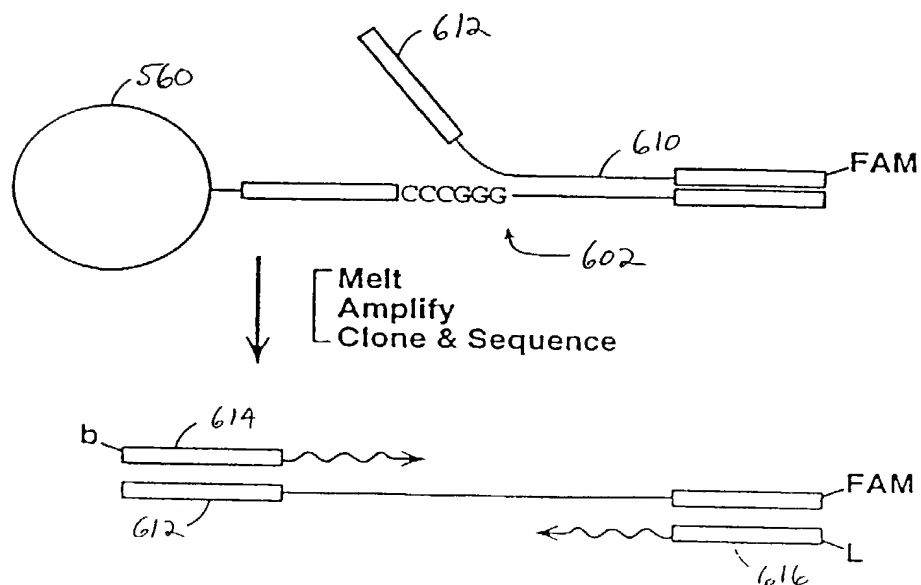
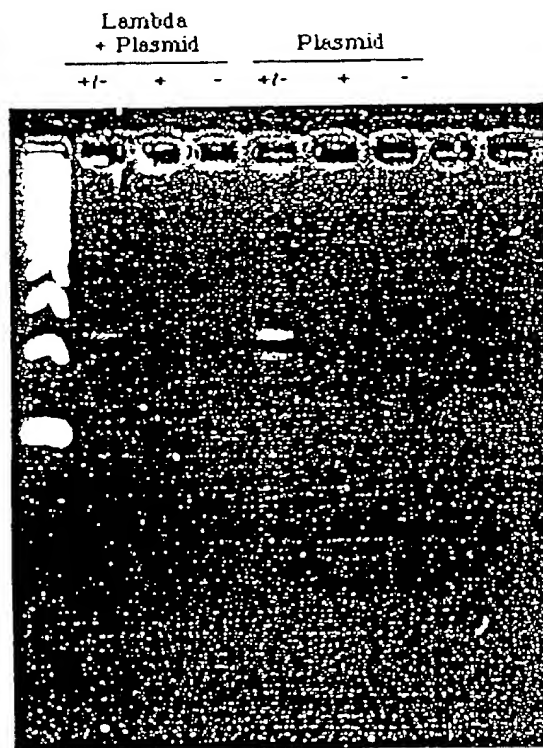
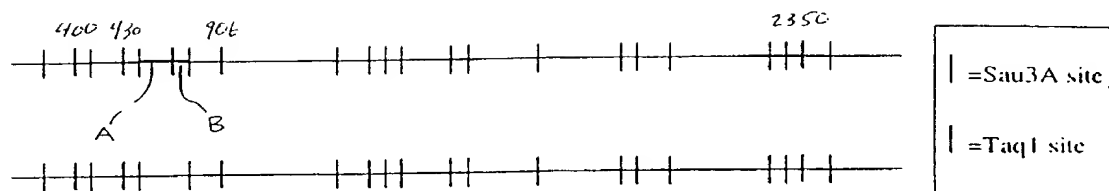


Fig. 6B

Fig. 7A



Expected bands approx 190bp
230bp

Fig. 7B

Generation of Taq+ fragments

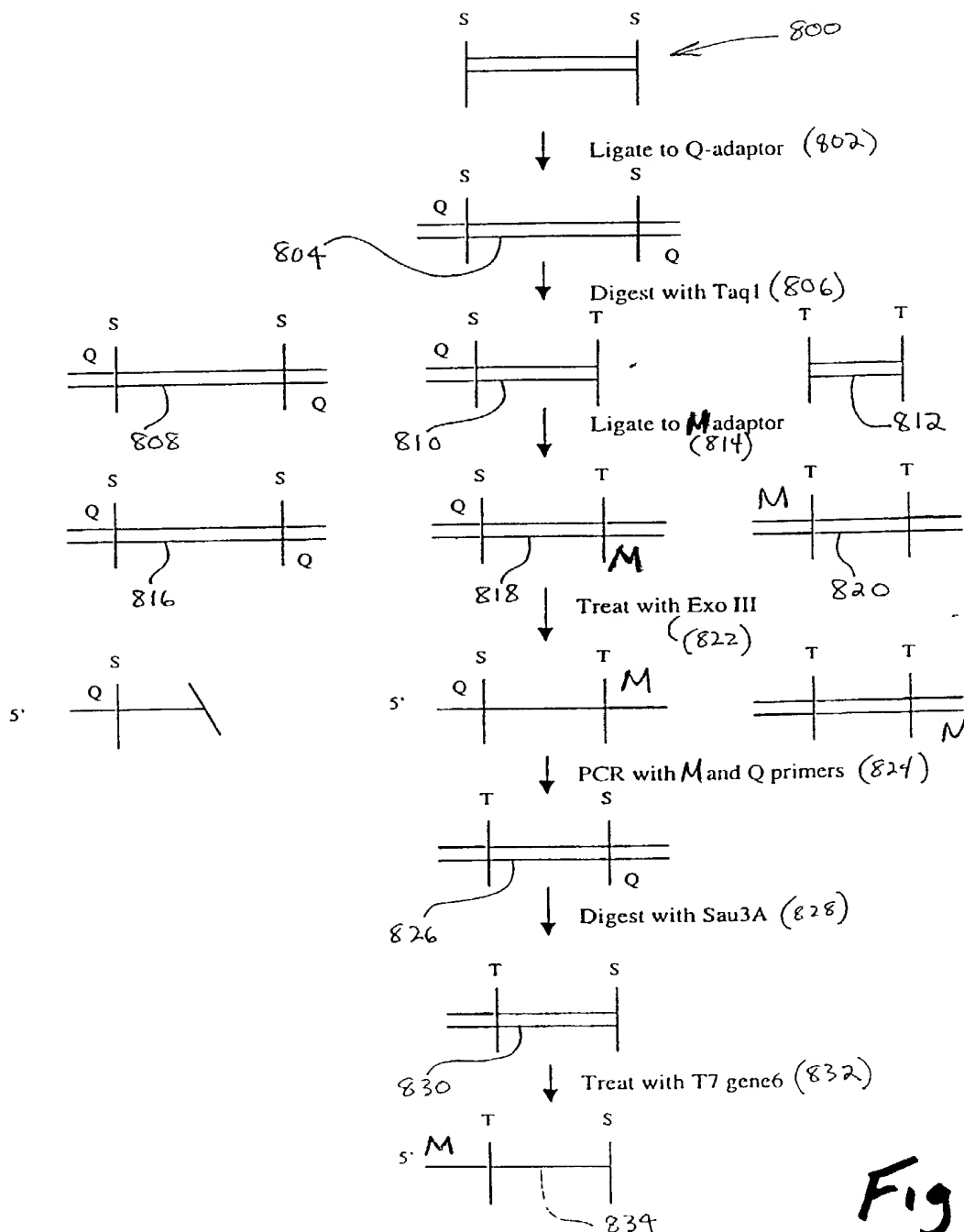


Fig 8A

Generation of Taq- fragments

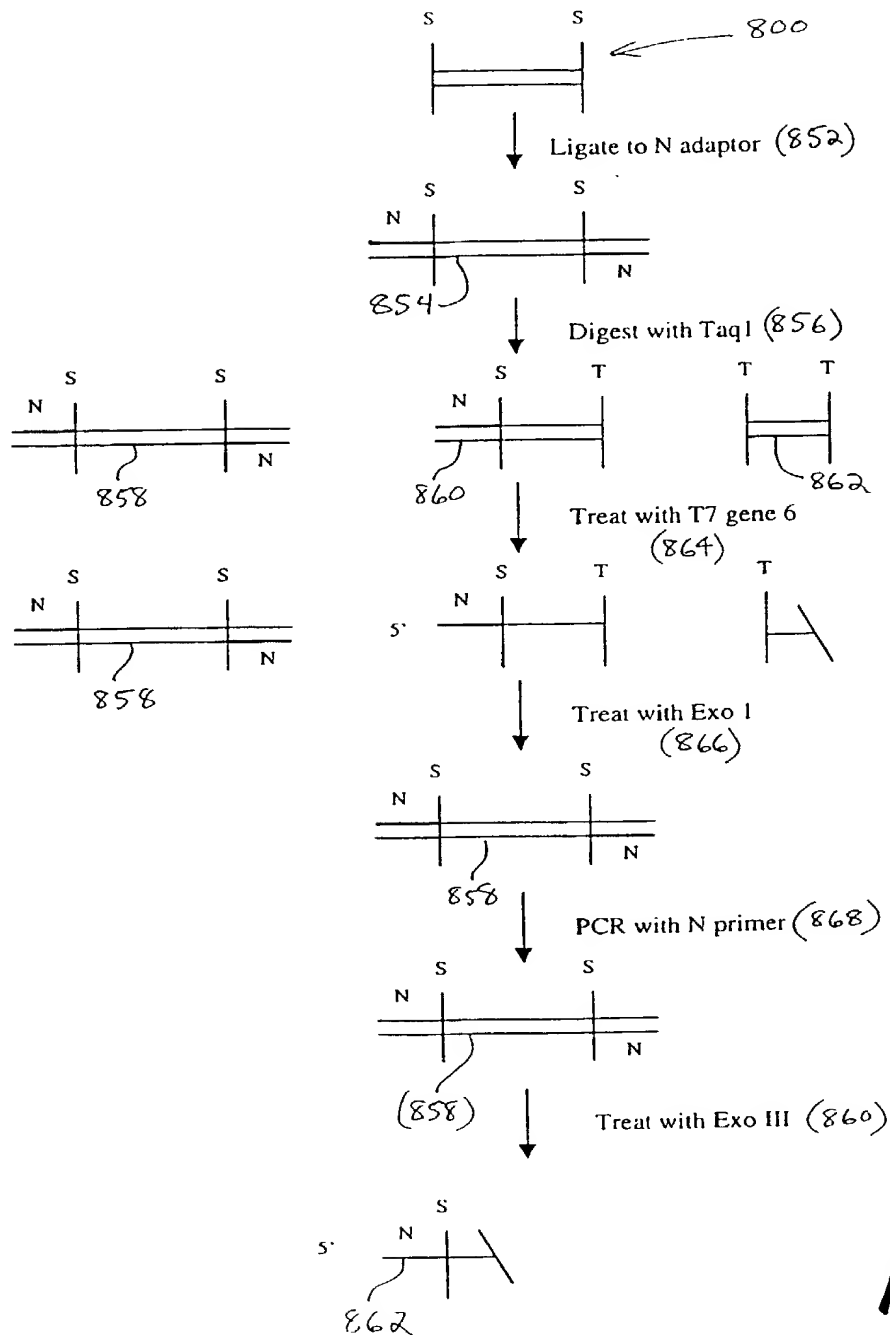


Fig 8^B

Rescue of polymorphic fragments

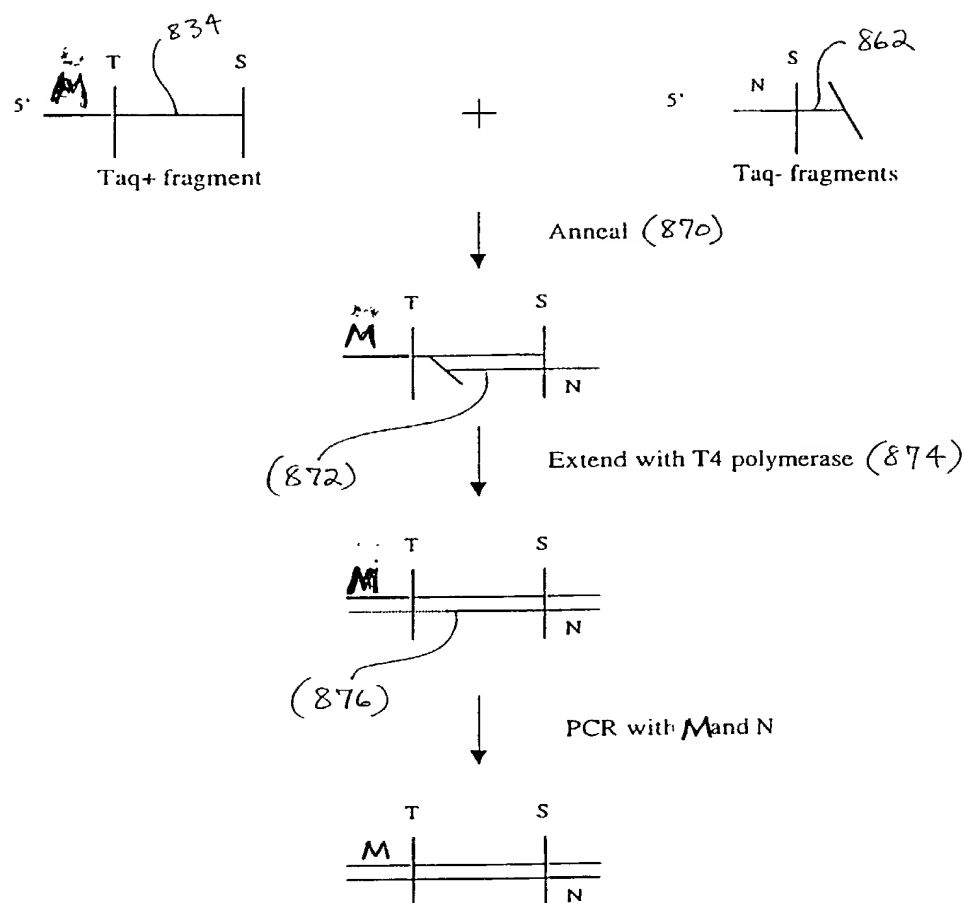


Fig 8C

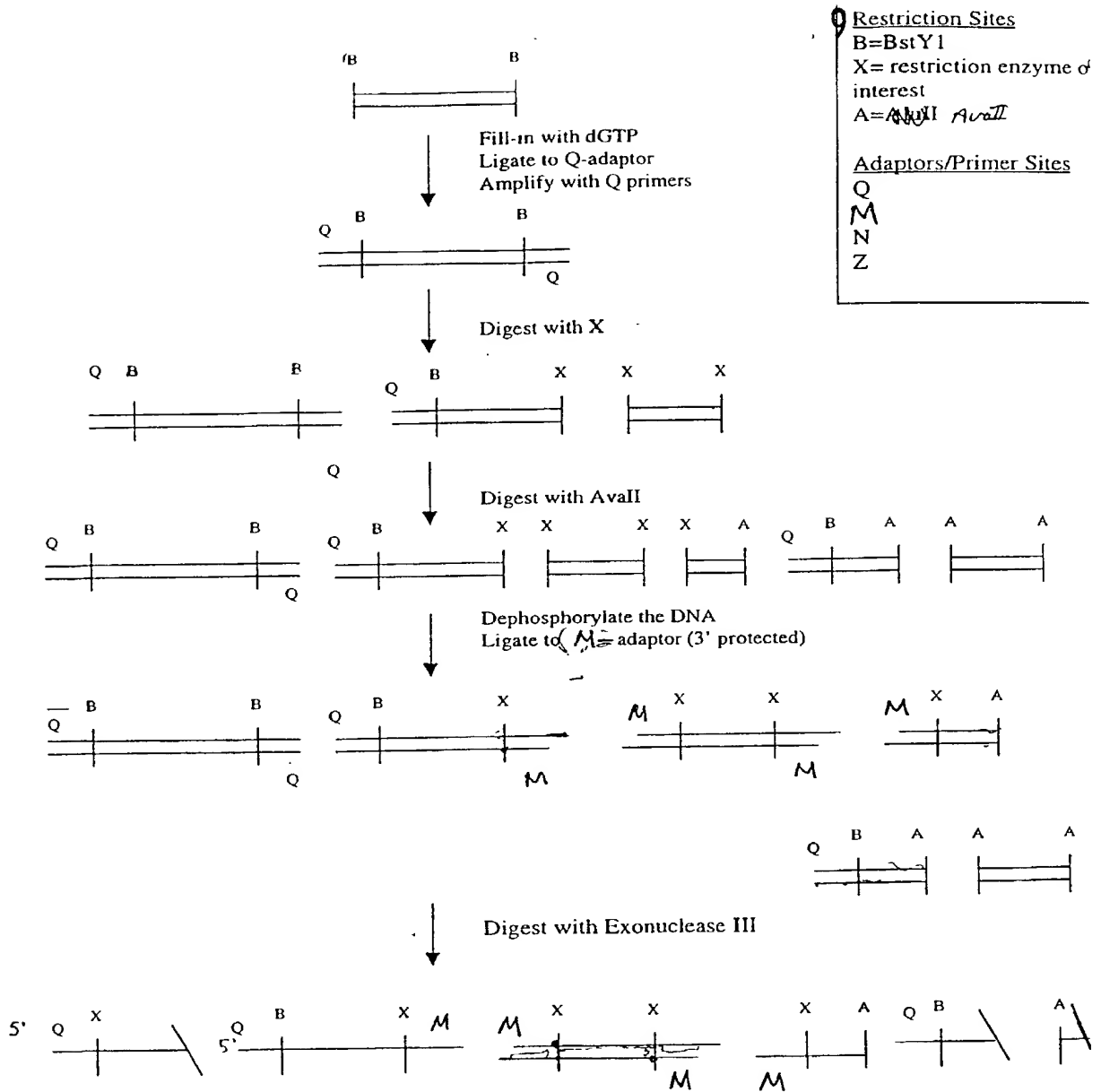


Fig 9A

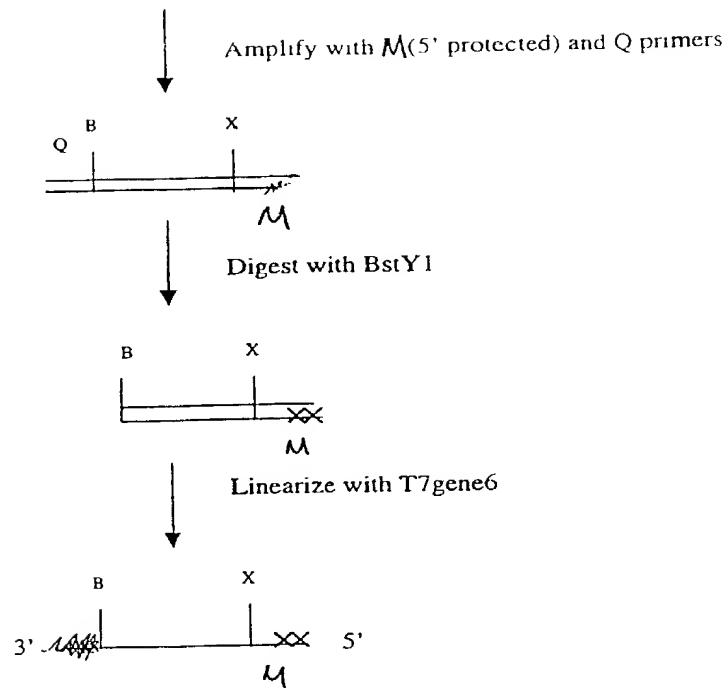


Fig. 9B

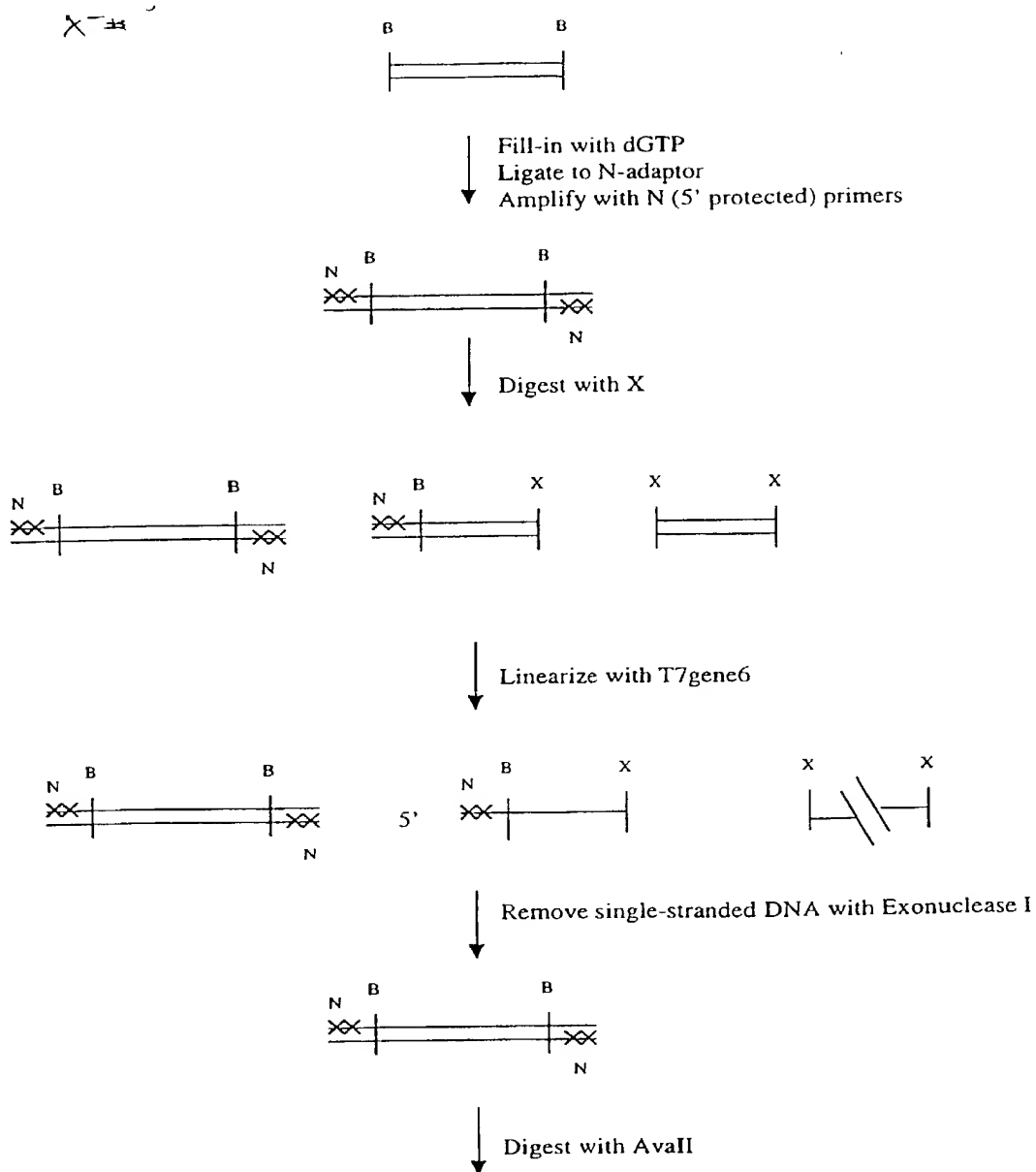
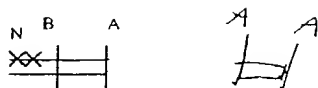
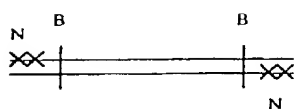


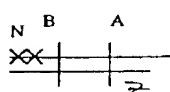
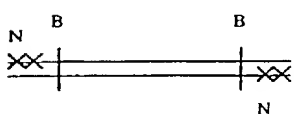
Fig 10A.

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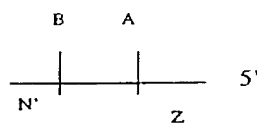
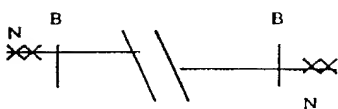
09/914101



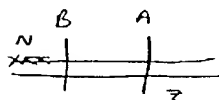
↓
Fill-in with ddGTP
Ligate to Z-adaptor (3' protected)



↓
Linearize with ExonucleaseIII



↓ PCR with N (5' protected) + Z-tag



↓ T7 gene 6 treatment

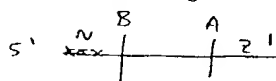
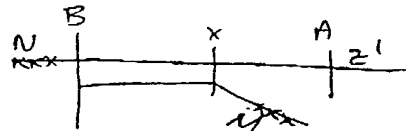
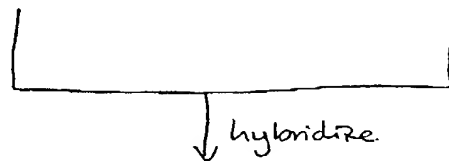
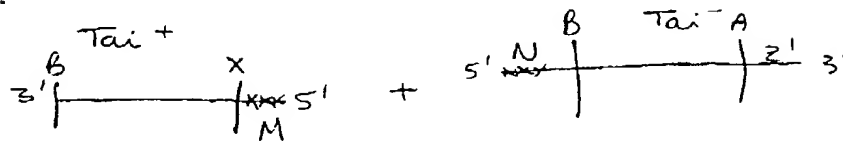
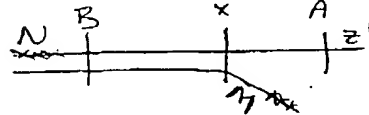


Fig. 10B

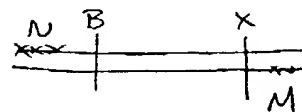
Figure 11

Hybridization of Tai^+ and Tai^- 

↓ extend with Taq DNA polymerase
or sequenase



↓ PCR with N + M primers



= reference SNP library

COMBINED DECLARATION AND POWER OF ATTORNEY
 (Includes Reference to PCT International Applications)

 ATTORNEY'S DOCKET NO.
5525-0044.10

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

POLYMORPHIC DNA FRAGMENTS AND USES THEREOF

the specification of which (check only one item below):

- ☐ is attached hereto.
☐ was filed as United States application Serial No. _____ on _____, and was amended on _____, (if applicable)
☒ was filed as PCT international application Number PCT/US00/04349 on 18 February 2000,

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) which designated at least one country other than the United States of America listed below and have also identified below, by checking the box, any foreign application(s) for patent or inventor's certificate, or of any PCT international application(s) having a filing date before that of the application(s) on which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC §119
			<input type="checkbox"/> YES <input type="checkbox"/> NO

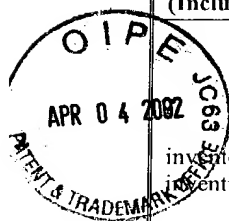
I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or 365(c) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT application(s) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56(a) which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120:

U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
60/158,483	08 October 1999			X
60/121,023	22 February 1999			X
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO.	PCT FILING DATE	U.S. Serial Nos. Assigned (if any)		
PCT/US00/04349	18 February 2000		X	

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Includes Reference to PCT International Applications)

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	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

SIGNATURE OF INVENTOR 201

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Date 17 March 2002

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